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EFFECTS OF ALTERNATIVE CARBOHYDRATE SOURCES AND SOYBEAN-
DERIVED ISOFLAVONES ON THE MICROBIOME AND IMMUNE SYSTEM OF
NURSERY PIGLETS AND INTESTINAL PORCINE EPITHELIAL CELL LINE J-2

By

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EFFECTS OF ALTERNATIVE CARBOHYDRATE SOURCES AND SOYBEAN-
DERIVED ISOFLAVONES ON THE MICROBIOME AND IMMUNE SYSTEM OF
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University of Nebraska, 2019

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Four experiments were conducted to characterize the microbiome of the piglet and observe effects of diet on growth performance and immunity. In Exp. 1, 96 nursery piglets were fed varying carbohydrate sources and in Exp. 2, 64 piglets were fed a soy protein concentrate or soybean meal as-is or supplemented with an isoflavone concentrate. Body and feeder weights were measured for growth performance estimation. Luminal contents and scrapings were isolated for microbial composition analysis. Fecal and blood samples were extracted in Exp. 2 for microbiome and immunity analysis. Diets did not affect growth performance ($P > 0.10$); however, Exp. 1 piglets had greater average daily feed intake of the experimental diets in phase 1 ($P < 0.05$). There were no differences in microbiome composition among diets ($P > 0.10$), but a trend over time ($P = 0.11$). Differences in microbial composition between sampling locations were shown in Exp. 1 and 2 ($P < 0.01$). In Exp. 2, diet and time altered microbial diversity ($P < 0.001$). IL-8 was not influenced by diet, ($P > 0.10$) but increased over time ($P < 0.001$). In Exp. 3, IPEC-J2 cells were treated with varying doses of the isoflavone genistein, lipopolysaccharides (LPS), or polyinosinic:polycytidylic acid (poly I:C). In Exp. 4, cells were treated with the isoflavones genistin, genistein, daidzin, or daidzein, and poly I:C or

diclofenac sodium salt. Media from the basolateral membrane or apical membrane sides were used for IL-8 ELISA and TEER was measured to estimate epithelial integrity. In Exp. 3, LPS showed greatest electrical resistance ($P = 0.014$) unlike Exp. 4 that showed no differences in TEER for time or treatment ($P > 0.10$) In Exp. 3 and 4, IL-8 concentration increased over time ($P < 0.01$) and was greater in the BLM ($P < 0.05$). In Exp. 4, IL-8 production from the poly I:C treatment was greater than the other treatments ($P = 0.0186$). In conclusion, characterization of the microbiome is influenced by diet, time and sampling location. Immune parameters were not different in the in vitro or in vivo trial with isoflavones and diet did not affect growth performance.

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DEDICATION

To my mother Teresa Kinstler and my father Jeff Kinstler

This is just as much your success as it is mine. I wouldn't be here today if it weren't for your sacrifices, your relentless love, and everything you've done to try to provide the best life possible. I haven't done anything to deserve parents like you, but I promise to live the rest of my life doing justice to all that you have done for me.

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Chapter 1: Literature Review

INTRODUCTION

Nutritional intervention is becoming a staple in the livestock industry with the new implementation of the Veterinary Feed Directive prohibiting the use of non-prescribed antibiotics in feed and leaving producers wondering how they might protect their livestock from disease. Gut health improvement has recently become a popular topic for preventing the spread of pathogens and is necessary as disease is the greatest contributor to economic losses in the livestock industry with the U.S. exporting \$6.32 billion in pork in 2016 alone (Schulz et al., 2016) and a concern for mortality in humans. Researchers and producers are striving to discover compounds found in common feed sources that could be isolated and supplemented to positively influence the gastrointestinal microbiota while minimizing the use of antibiotics. The integration of gut bacteria colonization and maturation of the immune system has been widely accepted as valuable in managing and preventing disease by using alternative diet ingredients to manipulate microbial composition and influence immune parameters as a less invasive method than antibiotic use (Filippis et al., 2018). Antibiotics have also been found to lower the immune system by deteriorating both beneficial and detrimental microbes, creating the need for other medicinal factors that could be implemented while leaving the microbiome intact. If the microbiome is able to sustain a healthy environment and enhance immune responses through nutritional intervention, the slower the onset of disease could be, providing researchers and producers more time to diagnose and treat disease before a devastating impact ensues.

Immunity is represented by the presence of microbes that are able to fend off invading pathogens and can be influenced by gut health as well as extrinsic and intrinsic

factors of the immune system. This can be measured both in vivo and in vitro by quantifying cytokine levels, T cell functionality, monocytes, and others. Researchers are interested in conducting experiments where adequate data are measured with the least cost and time, creating a demand for cell culture trials, including a cell line cultured from the jejunum of an unsuckled neonatal piglet. To determine if an experiment is worth the time, money, and effort put into a live animal trial, it's reassuring to first have a response in an in vivo trial.

The objective of this chapter is to review the development and composition of the microbiome, the immune parameters that may be studied, measured, or influenced, and discuss an epithelial cell line used in nutritional studies.

THE GUT MICROBIOME

Background

The gut microbiome consists of a dynamic microbial population that influences the phenotype of a mammal and represents the community of intestinal microorganisms. It historically was referred to as microflora, until non-bacterial elements such as archaea, fungi, and viruses that also regularly inhabit the gut were accounted for (Quigley et al., 2013). While it maintains a symbiotic relationship with the host, an imbalance of microbes, such as during times of stress, causes susceptibility to disease (Rea et al., 2016). Antibiotics have been used to combat symptoms; however, there has been concern that anti-microbial resistant bacteria has developed and resulted in a demand for decreased use of antibiotics by the public (Dibner et al., 2005), increasing the need for nutritional intervention. Diet of the host and microbes is critical to the digestion and

absorption of nutrients, contributing to the integrity of the intestinal epithelial layer that prevents pathogens from invading the host's gastrointestinal tract (Kyle et al., 2017).

Diversity follows patterns of community structure, but the microbiome is a unique influencer of gut health that can be managed through diet and environment.

Development

The development of the microbiome depends on environment and influences mammalian health and prevention of disease. The diversity of microbes, especially in young mammals, is crucial to avert autoimmune and inflammatory diseases that may result later in life if the microbiome is not properly constructed. One study has shown that growth and change in composition of gut microbes starts before the mammal is born by contribution of maternal microbiota to the fetus as the fetus initially maintains a sterile gastrointestinal tract (GIT); however, this could have been due to contamination of the amniotic fluid (Walker et al., 2017). As maturation occurs, the mammalian gut becomes home to over 100 trillion bacterial cells (Holman et al., 2017) and the majority of microbial development takes place in the first 2-5 years of life. The microbiome is influenced by the amount of exposure to a variety of microbes, pathogens, and antibiotics in everyday scenarios such as delivery method, environment, lifestyle, genetics, and diet (Rodríguez et al., 2015).

Most studies have shown that the GIT is first inoculated when the infant is exposed to maternal vaginal and fecal bacteria during birth. Mammals that experience assisted delivery that are not allowed as much exposure to diverse bacterial species have been shown to struggle with optimal colonization for the first few years of life (Jakobsson

et al., 2013). The first bacteria to populate the GIT in an anaerobic environment include *Proteobacteria*, *Prevotella*, and *Actinobacteria*, but the GIT changes to include higher abundances of *Bacteroidetes* and *Firmicutes* as age sets in (Rodríguez et al., 2015).

Germ-free animals show the importance of regular development of gut barrier microbiota and their functions, as they suffer from compromised immune systems and inhibited progress in the epithelium, vasculature, neuromuscular apparatus, and gut endocrine system when not exposed to typical microbial environments (Quigley et al., 2013). In the swine GIT, *Prevotella* is the most abundant genus in the adult, but is also commonly found in high quantities in humans and some ruminant animals. Colonization is still predisposed to change as a result of diet whether, the infant consumes bacteria naturally found in milk or if a formula is consumed. Over 700 species of bacteria are found in milk (Cabrera-Rubio et al., 2012) which stimulates colonization of *Bifidobacterium* that's originally introduced to the microbiome during birth. Infants have altered microbial composition compared to adults that gradually becomes similar as nutritional needs change, reflecting a necessity for different species. As solid food is introduced into the diet, butyrate producers such as *Bacteroides* and *Clostridium* become more prominent throughout adulthood (Neu et al., 2011). Microbiome studies depict similarity of microbial composition in all species, contributing to evidence that these studies are interchangeable.

Diversity

Studies focus on alpha- and beta-diversity of the microbiome that measure the presence of bacteria within individuals and the variation between individuals,

respectively. Diversity indices allow researchers to depict the differences in a community and point out rarity while quantifying diversity. As the microbiome is an ever-changing environment, diversity is highly variable over time and across human populations; however, there have been attempts to establish a core microbiome (Holman et al., 2017). The malleability of the microbial composition is crucial, as a more diverse setting will optimize colonization resistance where the native microbes prevent the invasion of pathogens and even beneficial microbes. A lower presence of species has been associated with dysbiosis in the gut and is associated with autoimmune diseases, cardiometabolic disease, and obesity (Sacks et al., 2006). In a rapidly fluctuating setting, diversity of microorganisms aids in maintaining regular functionality when environmental challenges arise and is most influenced during the early stages of life. Population abundance of microbes in the gut has been linked to metabolism, homeostasis, and immune development and can be altered by close contact with other individuals or material ingested (Maslowski et al., 2011). There is a need for more studies that characterize the microbial patterns in the environment to further understand the influences on the structure of the microbiome and how it becomes established for some microbes compared to others. In diet, it has been shown that omnivores have a higher diversity when compared to vegetarians, proposing that microbiota composition varies with diet (Maslowski et al., 2011). Understanding that difference in locations and cultures result in different eating habits and sources, microbial diversity varies across the world, even within the same species as the African microbiome diversity is greater than Western cultures due to further age and exposure (Conlon et al., 2014). Once established in the GIT, microbes become more stable as they find their niche and stake claim on the space and nutrients

needed for survival. A niche has been described as the complex description of how an organism uses its environment depending upon the metabolic properties of the organism and are constantly adapting to fill every ecological niche on the planet by fighting for resources that determine survival and genetic persistence (Bauer et al., 2018). Events that occur as a part of life, such as surgery or infections, can have detrimental impacts on diversity as the use of antibiotics can cause the deterioration of beneficial microbial populations while attempting to rid of the harmful organisms. The presence of certain macronutrients will influence microbiota as the more nutrients to thrive off of, the more competitive a species can become and colonize in the gut more efficiently. A study using high-fat diets saw an increase in *Rikenellaceae* in turn for a decrease in *Ruminococcaeae*, a prominent carbohydrate fermenter (Jakobsson et al., 2013). The availability of nutrients creates an advantage for some species by competition in the GIT.

Relationship with the host

The relationship between the microbes that inhabit the gut and their host is highly complex and health and disease of the host depend on microbial diversity. The development of the GIT microbiota occurs in parallel to the growth, maturation, and sprouting of neurons in the young brain (Rea et al., 2016). The microbiota-gut-brain axis is a matrix of tissues that communicate to regulate various immune functions based on microbial composition in the gut (Rea et al., 2016). The microbes communicate with the host by influencing the enteric, sympathetic, and parasympathetic nervous systems to activate neuroimmune components of the central nervous system. This close relationship is mutually beneficial and contributes to the host's energy regulation, gut barrier function,

pathogen protection, and overall immune system function (Bengmark et al., 2013). The human GIT microbiota alone upregulate nearly 2 million genes (Quigley et al., 2013). Communication between commensal bacteria and the host induce regulatory cells, such as T cells, that consequently produce anti-inflammatory cytokines for the animal (Conlon et al., 2014).

Diet in microbes

Microorganisms are most influenced by macronutrients in the diet and GIT. Depending on the presence of starches, fibers, proteins, and other dietary components ingested, variation in individual microbial composition can be as unique as a fingerprint.

Carbohydrates

Carbohydrates are the key carbon and energy sources for microbes, metabolized into short-chained fatty acids (SCFA) during fermentation (Conlon et al., 2014). Among these SCFA is butyrate that is synthesized by microbes and serves as the principal source of metabolic energy for cells located in the colon, or colonocytes. Carbohydrate digestion begins in the mouth with enzymatic degradation by salivary amylase. After carbohydrates pass through the esophagus into the stomach and enzymes are inactivated in the acidic environment, a majority of the carbohydrates enter the small intestine. The mammalian genome does not encode a large enough quantity of the enzymes required for carbohydrate degradation, signifying the need for microbial fermentation using carbohydrate active enzymes, or CAZymes (Flint et al., 2012). The prominent families of *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* possess the greatest quantity of CAZymes

that degrade specific polysaccharides, depending upon their location in the GIT, as shown in Table 1. *Firmicutes* and *Bacteroidetes* pathways are specific to fructan, lactose, and starch metabolism and the CAZymes increase degradation of these carbohydrates in the extracellular space, cytoplasm, and periplasmic space (Wang et al., 2019). The microbiota show flexibility in that they're able to transition between metabolizing diet and host-derived carbohydrates, proving they're not limited to metabolizing only waste after physical and chemical degradation by the host. The structure of the carbohydrates influences microbial composition because the bacterial community depends on the availability of simple carbohydrates (Zoetendal et al., 2012). If non-digestible carbohydrates are excluded from the diet, the microbiota will utilize endogenous energy sources, including mucin that lines the intestine, which results in the degradation of the membrane that serves as epithelial cell protection and increases risk for pathogen invasion. While simple starches are readily utilized in the small intestine, resistant starches are the largest sources of energy for microbes provided by diet, depending upon the degree of processing of the carbohydrate source before the feed is ingested. The host and microbiota both depend upon carbohydrates for energy to use in cellular processes and for growth by the degradation of complex carbohydrates in cell walls and starches.

Protein

Bacteria and the host provide proteases and peptidases to hydrolyze proteins into peptides and amino acids in the small intestine. These metabolites can be further utilized by the host and the microbes, feeding off of one another. *Clostridium*, *Lactobacillus*, *Streptococcus*, and *Proteobacteria* are amongst the leading fermenters of amino acids in

the small intestine (Cho et al., 2007). *Clostridia* and *Peptostreptococci* manage the amino acids that reach the large intestine; however, if too much protein fermentation occurs when these residuals reach the colon, cytotoxic metabolites such as ammonia and carcinogens can be detrimental to the microbial population if the nitrogen isn't utilized by the microbes (Conlon et al., 2014). The process of degradation in the small intestine is by bacteria containing highly active peptidases is extremely efficient; however, not all metabolites are utilized in this location and will either enter the bloodstream or continue into the colon to be used by colonocytes. The amino acids utilized undergo *de novo* synthesis to produce other amino acids, such as lysine, particularly in the large intestine where microbes harbor a voluminous number of genes used in essential amino acid biosynthesis (Sacks et al., 2006). Amino acids may also be used by anaerobic bacteria as a precursor for the SCFA acetate and butyrate. Another SCFA, propionate, is exclusive to synthesis from threonine, providing alternative energy sources to carbohydrates. This is a significant consideration for trials exploring carbohydrate sources, as the protein content must be similar.

Lipids

The digestion of lipids by microbiota is less clear as a majority of studies assumed that fat was digested mainly in the small intestine. It has been shown that microbes only influence certain aspects of lipid metabolism, including body mass index as well as composition of triglycerides and high-density lipoproteins. Most fats are regulated by bile acids present in the small intestine, and facultative and anaerobic bacteria produce secondary bile acids that subsequently impact glucose metabolism once immersed in the

bloodstream (Ghazalpour et al., 2016). These bacteria also participate in complex carbohydrate fermentation, influencing the production of SCFA that influence immunity and have hormonal effects in the host. The microbiota also produce fatty acid oxidation precursors that enhance lipid metabolism, such as trimethylamine N-oxide (Ghazalpour et al., 2016). The role of microbiota in lipid digestion is less clear than other macromolecules and further experimentation is needed to determine the effect that GIT microbes have on fat and vice versa.

Gut health in relationship to disease

Understanding that providing a complete diet to not only the host, but the microorganisms in the gut influences immune functions, it's apparent that the welfare of both the microbiome and the host depends on optimal gut health. Microbes regulate stress, the disturbance of the homeostasis of an organism, preventing neuroinflammation that has been attributed to psychiatric disorders such as anxiety and depression (Koonin et al., 2012). During times of stress, a loss in lactic acid producing bacteria is observed, which results in an increase of pH. This results in enhanced disease susceptibility as a low pH is crucial in the gut to maintain a bacteriocidal environment that invading pathogens cannot normally survive in. If disease is on setting, the host and microbiome may also develop a negative relationship where the host cannot recognize the normal flora and an inflammatory response ensues that damages the epithelial lining and results in bacteria entering the intestinal wall, submucosa, and circulation (Quigley et al., 2013). Maintaining positively progressing gut health can prevent pathogen invasion by regulating normal bodily functions and maintaining homeostasis by use of the beneficial

metabolites supplemented by microorganisms in the GIT. When antibiotics are introduced to the microorganisms, they eliminate not only the problematic bacteria, but the beneficial microbes as well, resulting in a clean slate in the GIT for different pathogens to enter cause disease (Quigley et al., 2013). A situation that compromises pathogens without disturbing the entire microbiome could therefore enhance the immune response of the host by maintaining the desired microbial responses to disease.

SOYBEAN-DERIVED ISOFLAVONES

Background of isoflavones

Isoflavones are antinutritional factors that have recently gained attention for their estrogenic properties and effects on hormone-dependent and age-related diseases, such as cancer. Isoflavones are a class of flavonoids, polyphenolic compounds commonly found in plants and fungus that are sought after for their antioxidant, antimicrobial, anti-carcinogenic, and anti-inflammatory properties. Found in legumes and soy foods, their structures resemble estrogen, thus binding to estrogen receptors in mammals to apply either a weak estrogenic or antagonistic effect. Hormonal regulation by the host can decrease estrogenic binding to receptors, depending on potency of other estrogen factors. Besides exerting estrogenic and antiestrogenic properties depending upon dosage, isoflavones are also protein tyrosine kinase (PTK) inhibitors (Andres et al., 2007). Genistein and daidzein are the bioactive forms that are cleaved by β -glucosidases in the gut microbiota and host cells during digestion from the zymogens genistin and daidzin. (Chen et al., 2005). While not all soy products have the same quantity of isoflavones due to processing methods, soybean meal, a feed ingredient regularly used in livestock diets,

contains a modest amount that could be further supplemented to attempt to regulate hormonal processes and enrich the gut microbiota.

Bioavailability and metabolites/digestion

Isoflavones are the most well utilized of the flavonoid family and genistein has been proven to possess the greatest bioavailability (Yu et al., 2016). An unrefined soybean will contain between 26-381 mg/100 g of isoflavones that gradually decreases with degradation from processing, such as when heated. When ingested, soy isoflavones are primarily found in the glycosidic form (daidzin, genistin, and glycitin) before β -glycosidases in bacteria cleave the glycosidic groups to their aglycosidic form (daidzein, genistein, and glycitein) in the jejunum, making them bioactive (Aglin et al., 2013). Once activated, the isoflavones are either absorbed intact or further metabolized by the intestinal microbiota to metabolites such as *O*-glucuronides, equol, and sulphate esters in the gut barrier before metabolism in the liver (Zubik et al., 2003). *S*-equol, a non-steroidal estrogen metabolized from daidzein, is only produced by 30-50% of the bacterial population and is a potent antioxidant that binds to estrogen receptors with greater affinity than daidzein (Frankenfeld et al., 2011). This occurs mainly when isoflavones are not hydrolyzed or absorbed in the intestine and enter the colon. Genistein is metabolized into dihydrogenistein by bacteria and daidzein is metabolized into dihydrodaidzein, as shown in Figure 1. Once daidzein becomes 5-hydroxy-equol or generic equol, these metabolites will bind to the estrogen receptors, particularly ER- β (Anupongsanugool et al., 2005). Isoflavones may be absorbed and exert hormonal activity effects at any point of digestion, but are dependent upon intestinal microbiota composition for the extent of

metabolism and determining which metabolites are formed. When configuring bioavailability and concentration of isoflavones in samples after digestion, it must be taken into consideration that the glycosidic forms will be reduced as a consequence of the β -glucosidase activity.

Anti-viral and anti-inflammatory properties

Inflammation is a biological response to harmful stimuli and control is tightly regulated to prevent the over-reaction of the innate immune response. Isoflavones are well-known for their capabilities to battle disease through their antioxidant, antimicrobial, and anti-inflammatory properties both in vivo and in vitro. Isoflavones have been proven to down-regulate cytokine-induced signal transduction and reduce leukocyte numbers in the blood while decreasing IL-6, nitric oxide (NO), and prostaglandin E₂ (PGE₂); (Chinta et al., 2012). Inhibition of pro-inflammatory genes, such as COX-2 by genistein, propose that the suppression of mediators that induce undesirable responses will delay the onset of diseases, such as Parkinson's Disease, with increased soy consumption. Daidzein inhibits oxidative induction of the MAP kinase-NF κ B signaling pathway that mediates inflammatory responses and is a key factor in pathogenesis of several diseases (Lamartiniere et al., 2002).

Antiviral properties are most elicited by genistein to inhibit the infectivity of a range of DNA and RNA viruses. Isoflavones also influence cell membrane structures and regulate interactions with tight junction multiprotein complexes that are activated during stress to prevent invading pathogens from entering the enterocytes of the small intestine. Additive immune properties from the consumption of soy-based infant formulas decrease

rotavirus infection that causes performance loss through upset stomach that results in diarrhea and vomiting in young mammals (Andres et al., 2007). Dietary supplementation induces immune responses by activating factors such as cytokine production from T cells that mobilize neutrophils to sites of infection, inhibiting natural killer cell cytotoxicity that are crucial to the innate immune system, and even the inhibition of IgE receptor signaling that results in the prevention of allergies (Sakai et al., 2008). When activated, genistein has been identified as a potent inhibitor of protein tyrosine kinase (PTK) activity associated with epidermal growth factor receptor (Brosnahan et al., 2012). PTKs regulate cellular activities, such as cell division and in some cases, can be mutated and result in uncontrollable cell division, resulting in cancer. Genistein has been shown to inhibit DNA topoisomerase II, an enzyme that regulates DNA overwriting and underwriting, and the prevention of these actions results in permanent DNA strand breaks that overwhelm the cellular processes for repair and initiate apoptosis instead (Andres et al., 2007). More experimentation focusing on the mechanisms of the antiviral properties are of interest as it has not been confirmed that PTK inhibition, DNA topoisomerase II inhibition, and timing of exposure to isoflavones have an effect on infectivity of pathogens. It is suspected that the antiviral mechanism is related to a binding or postbinding event in viral cell entry and replication.

Impact on microbiome

There is a dependence on the microbiome to perform metabolism, thus emphasis is made on the intestinal bacterial profiles of the consumers to determine efficiency; however, some phenolic compounds, such as isoflavones, have been found to be

antimicrobial. The baseline microbiome composition dictates how polyphenols are metabolized because both beneficial bacteria and pathogens are predisposed to isoflavones and their inhibitory effects. Some studies have contributed the contradictory effects of isoflavones on the bioavailability to produce equol, but recent evidence relates these effects to the presence of sulphate reducing bacteria (Nakatsu et al., 2014). Few studies have focused on the characterization of the microbiome with soy intake, but most identified an increase in *Eubacterium* and *Bifidobacterium* (Paul et al., 2017).

Bifidobacteria is known to thrive off of isoflavone metabolites and it has been observed that *Clostridium* is higher in abundance in individuals capable of producing equol (Bolca et al., 2007). It would be of interest to examine human microbial populations in Asian countries where soy is a common dietary component compared to the average Western diet that commonly excludes soy and isolate the bacteria more abundant in the Asian microbiome and determine which are capable of producing equol. Further experimentation is needed to characterize the microbiota that suffer or flourish as a result soy consumption to better understand which cultures or environments are ideal for isoflavone supplementation.

In relation to cancer and infertility

Firm effects of isoflavones on cancer are yet to be determined; however, studies show that higher malignancy incidences are related to S-equol binding to β -estrogenic receptors (Anupongsanugool et al., 2005). 6'-Hydroxy-*O*-demethylangolensin (6'-OH-DMA) is a metabolite of genistein that has stronger estrogenic properties than the parent

compound and can be absorbed by the intestinal mucosa or the liver (Matthies et al., 2008). 6'-OH-DMA is conjugated to *O*-DMA glucuronide and *O*-sulfate before released into circulation or bile (Frankenfeld et al., 2011). In a more targeted approach, it was discovered that a decrease in 17 β -estradiol glucuronidation resulted in an increase in risk of breast cancer development with excess consumption of isoflavone supplements in patients diagnosed with ER α breast cancer (Dong et al., 2010). Cancer is a pleiotropic disease and genistein is a pleiotropic compound, giving rise to opportunities for undesirable events to transpire when combined. There are cases that show that the introduction of isoflavones into cancer patients causes cell proliferation, accelerating cancer invasion (Kurzer et al., 2000). These results depend on the health status, age, sex, and the presence or absence of specific gut microflora as only some populations are able to metabolize the phytoestrogens and are not as abundant in Western cultures. Most contribute the adverse effects and increased cancer cell proliferation on the type of estrogen receptor more prominent in the individual and if the patient is pre- or post-menopause, as women harboring more ER- α receptors that are post-menopausal are more prone to the undesirable effects (Russo et al., 2016). Impaired fertility is another downfall that has resulted from the use of isoflavones. In the neonatal mouse model, mice could not deliver live pups after subcutaneous genistein exposure, showing that estrogen's mechanism in the female reproductive tract is mediated through ER- α (Barnes et al., 2011). In premenopausal women, isoflavones decrease midcycle luteinizing hormone and follicle stimulating hormone concentrations. These side effects impair growth of ovarian follicles before the release of eggs (Kurzer et al., 2000). While women experienced a decrease in breast cancer risk as a result of an increase in menstrual cycle length and

decreased urinary estrogen excretion, these side effects are both indications of lower fertility rates. In male subjects, mature sperm decrease when exposed to a high dietary dose of genistein and reduce testis and epididymis indexes, lactate dehydrogenase, and overall testosterone levels, negatively effecting reproductive function (Yuan et al., 2012). Adverse effects on reproduction and in cancer patients raise concerns for the use of dietary isoflavones and leave researchers wondering if the beneficial effects outweigh the detrimental outcomes. The question remains how to obtain the advantageous results with lowered risk for the unfavorable outcomes. These situations represent the need for individual characterizations and studies because there are many factors that contribute to the hormone therapy that ensues with isoflavones.

IPEC-J2 CELL LINE

Background

IPEC-J2 cells are a columnar intestinal porcine enterocyte cell line that are cultured from the epithelium of the jejunum in a unsuckled neonatal piglet. They were originally cultured by Helen Berschneider at the University of North Carolina in 1989 to examine transepithelial ion transport and proliferation (Berschneider et al., 1989). They are unique in that are not transformed, meaning they have a slower growth rate and lower plating efficiency than transformed cell lines and are derived from small intestinal tissue (Chopra et al., 2010). Small intestine cell lines have far less evidence when compared to the colon, even though these cell lines are highly resistant to injury and when harmed, apoptosis swiftly occurs and sloughs off the dead cells into the lumen. There is interest of the epithelial monolayer in the gut as it serves as a physical barrier to prevent harmful

microorganisms from invading the mucosa and averts infection. Thus, the functional integrity of the epithelial cell lines must be able to combat the multiple mechanisms of invasion by pathogens. IPEC-J2 are quickly becoming a prominent in cell culture studies due to their integrity, use in numerous assays, and ease of handling.

Characterization

A polarized monolayer is formed when IPEC-J2 cells are cultured and the use of fetal bovine serum (FBS) in the culturing medium as opposed to porcine serum results in high transepithelial electrical resistance (TEER) to form (Vergauwen et al., 2015). The high TEER is useful when investigating tight junctions and low active transport rates when challenged with compounds that are harmful to monolayer permeability. Tight junction proteins define barrier and permeability functions of the paracellular pathway and claudin-3 and -4 and occludin have been found in the apicolateral membrane (AM), resembling normal jejunum properties (Schierack et al., 2003). IPEC-J2 contain microvilli on the apical membrane side, imitating extension into the lumen and are characterized as a continuous cell line with studies using passages up to 98 (Vergauwen et al., 2015), proving great longevity. The characterization of the IPEC-J2 cell line for pathogenesis studies only took place in 2003 by Schierack; however, this cell line has shown the capability to possess very similar properties to human physiology and shows specificity to porcine-derived infections, proposing that these cells are viable for viral studies including both human and porcine pathogens (Boyen et al., 2009).

Immunity studies potential

IPEC-J2 cells are similar to human cell lines in that they secrete cytokines, defensins, and toll-like receptors that all participate in communication between the enterocytes and the innate immune system from pathogen recognition receptors. Cytokines, such as TNF- α , are secreted by IPEC-J2 proteins and mRNA expression of Toll-like receptor(TLRs)-2 through -10 and IL-1 α , -1 β , -6, -7, -8, -12A, -12B, and -18 have all been confirmed in these cells (Brosnahan et al., 2005). Presence of protein expressions of claudin-1, -5, -7, -8 -12, and tricellulin have also been established by immunoblotting (Zakrzewski et al., 2013), telling that a wide range of experiments may transpire given the number of variables that can be investigated. These properties have already been proven to be valuable in characterization of epithelial cell interactions with enteric bacteria and viruses and make IPEC-J2 cells an ideal cell line for investigating pathogens (Brosnahan et al., 2012). Similar responses in reference to immune parameters, such as IL-8, have been observed in IPEC-J2 cells comparable to a human cell line, colonic T84 cells, in response to infection (Vergauwen and Tambuyzer et al., 2015). Advantages of using IPEC-J2 cells as a model for normal intestinal epithelial cells include their consistency of differentiation characteristics that strongly mimic primary intestinal epithelial cells and are directly analogous to the experimental animal that is used as an in vivo model for humans. While originally purposed to determine ion permeability, IPEC-J2 cells have developed into the most similar cell line of non-human origin that can be referenced into human physiology.

SUMMARY

The gut microbiome is becoming strongly of interest for determining and influencing health and immunity. Manipulation of the microbial communities in the GIT will affect the digestion and absorption of nutrients from diet that maintain intestinal epithelial integrity and contribute to health. The symbiotic relationship between host and gut bacteria enable access to nutrients that are not typically available from cellulose and other fibrous compounds. The better the microbiome is understood, the more readily available nutrients can be delivered to maximize efficiency while determining the metabolites formed, such as those produced by isoflavones. While isoflavones have had adverse effects on specific bacteria with antimicrobial properties, the anti-inflammatory, antiviral, and antioxidant potential are highly sought after, and the detrimental effects could be masked when isoflavones are used appropriately. They exert estrogenic activity on the host while their metabolites prove to administer an even harsher effect against pathogens and disease. To take advantage of these properties, an accurate estimation of composition of the gastrointestinal microbiota must be determined for an individual and which estrogen receptor present in a higher quantity identified for accurate dosage administration of the isoflavones. While this appears to be an individualized process, experimentation to gauge the typical abundance of isoflavone metabolizing bacteria in an individual will allow for estimation of what could be an industry standard. These experiments could take place in an in vivo model but are recommended in vitro first because many immune parameters and gene expressions can be measured in a cell culture experiment before use in live animals. The integrity of the IPEC-J2 cell line and the numerous measurements that can be taken from this intestinal epithelial model are

applicable to observe several immune responses that are highly reproducible. IPEC-J2 cells are robust and could be used for disease challenges to examine how the epithelial lining reacts during times of stress and establish a baseline response of small intestine epithelial cells to a pathogen. Gut health is a trending topic in the scientific community; however, there is still much to characterize before manipulation of the microbiota to optimize health and growth performance can occur. Soy isoflavones and their conflicting results are a potential solution to the onset of disease; however, further explanation of the mechanisms involved in immunity are needed.

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TABLE AND FIGURE

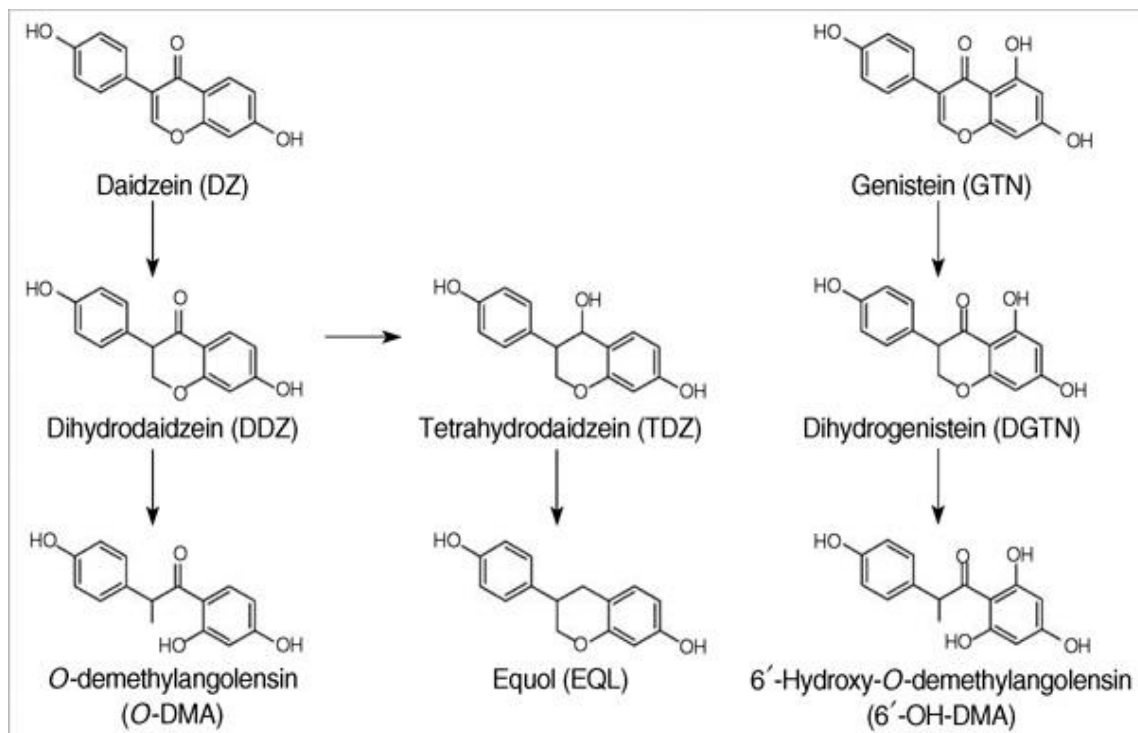
Table 1. Predicted CAZymes encoded by the genomes of selected fibrolytic gut bacteria
(Flint et al., 2012).

Ecosystem	Phylum (family)	Bacterium	Total CAZymes	GH	GT	PL	CE	Total CBMs
Human colon	Bacteroidetes	<i>Bacteroides thetaiotaomicron</i> VPI-5482	386	263	87	16	20	31
		<i>B. xylanisolvens</i> XB1A*	349	224	81	22	22	26
		<i>B. vulgatus</i> ATCC-8482	279	177	78	7	17	18
		<i>B. fragilis</i> 638R	223	138	78	1	6	26
	Firmicutes: (<i>Lachnospiraceae</i>)	<i>Roseburia intestinalis</i> XB6B4*	175	115	46	0	14	11
	(<i>Ruminococcaceae</i>)	<i>Butyrivibrio fibrisolvens</i> 16/4*	115	75	37	0	3	31
	Actinobacteria	<i>Ruminococcus champanellensis</i> 18P13*	87	54	12	9	12	34
		<i>Bifidobacterium adolescentis</i> ATCC15703	94	54	37	0	3	6
Rumen	Fibrobacteres/ Acidobacteria	<i>Fibrobacter succinogenes</i> S85	183	100	54	12	17	73
	Bacteroidetes	<i>Prevotella ruminicola</i> 23	215	133	60	3	19	16
		<i>P. bryantii</i> B14	203	107	53	14	19	un
	Firmicutes: (<i>Ruminococcaceae</i>)	<i>Ruminococcus albus</i> 7	145	96	24	7	18	128
		<i>R. flavefaciens</i> FD1	140 +	101	un	13	26	68

*For these strains, data were provided by the Pathogen Genomics group at the Wellcome Trust Sanger Institute and can be obtained from <http://www.sanger.ac.uk/resources/downloads/bacteria/metahit/>

The information presented is available from the CAZY website, except in the case of *R. flavefaciens* FD1^{27,30} and *P. bryantii*.⁴⁴ Un = information not available.

Figure 1. Metabolic pathway of bioactive isoflavones daidzein and genistein (Kwak et al., 2009).



Chapter 2: Effects of carbohydrate source on performance and gastrointestinal microbiota in nursery pigs.

ABSTRACT

An experiment was conducted to determine the effects of carbohydrate source on growth performance and gastrointestinal microbiota in nursery pigs. Ninety-six pigs weaned at 21 d were randomly allotted to 16 pens (6 pigs/pen; 4 pens/treatment) with sex represented evenly in each pen. Pigs had ad libitum access to feed and water for the 2-phase nursery period (28 d). Dietary treatments included: 1) Nutrisure™ (a food-grade cooked cereal grain product), 2) oatmeal (food-grade oatmeal grain), 3) steam-rolled oats (SRO), or 4) dried-distiller's grains as a negative control (DDGS). Ileal and colonic digesta and mucosa samples were collected on d 0, 14, and 28 (n = 6, 32, and 32, respectively) for analysis of microbial species using Illumina™ next generation sequencing. The DNA sequences were filtered through the DADA2 pipeline, Mothur, and QIIME. Pig body weights and feeder weights were also measured on d 0, 7, 14, 21, and 28. Pigs fed DDGS had lower average daily feed intake (ADFI) compared to other treatments during phase 1 ($P < 0.05$), but there were no differences in average daily gain (ADG) or gain:feed (G:F) among treatment. There were no differences among treatments for ADFI, ADG, or G:F for phase 2 or the overall experimental period. Microbial composition differed significantly among location in the gastrointestinal tract ($P < 0.01$) and among the digesta and mucosa samples extracted from the ileum and colon. *Lachnospiraceae pseudobutyrvibrio* and *butyrvibrio* were identified as the species primarily responsible for variation in sample location ($P < 0.01$). There were no significant changes in the microbiota among any treatment ($P > 0.10$). Over time, there was a trend ($P = 0.111$) for microbial diversity to vary; however, there was no interaction among diets or as a result of sampling time point. In conclusion, carbohydrate sources did

not affect gastrointestinal microbiota composition, ADFI, ADG, or G:F overall in this experiment. However, feed intake during phase 1 differed between DDGS and oat-based carbohydrate sources and microbial species were different between digesta and mucosa in the ileum and colon.

Key words: carbohydrate sources, gastrointestinal microbiota, growth performance, nursery pig

INTRODUCTION

Carbohydrates are the primary source of energy for the gastrointestinal microbiota and are used in the production of short chain fatty acids (SCFA) in the bowel as fermentation products from food components that are not absorbed or digested in the small intestine. Diet has been shown to affect gut microbiota composition and impact human and swine health while preventing disease progression (Singh et al., 2017). Bacteria in the upper intestinal tract are able to metabolize simple carbohydrates while the latter portion includes bacteria that ferment the complex carbohydrates to break down the more complicated β -1,4 glycosidic bonds found in cellulose, a compound found in cell walls in plants (Zoetendal et al., 2012). In order to enhance immune system response, energy for biological processes is needed, suggesting there is reliance on the carbohydrate structure and source ingested by the host and utilization capabilities of the gut microbiota (Round et al., 2009). SCFA strengthen the mucosal barrier and affect immunity by inducing responses in toll-like receptor (TLR) expression, antigen presenting cells, differentiated T cells, among others (Flint et al., 2012). The microbiome has the ability to affect diseases such as inflammatory bowel disease where there is less bacterial diversity with an exclusion of prominent carbohydrate digesting bacteria such as *Bacteroides* and *Firmicutes* (Singh et al., 2017). Evidence shows that *Bacteroides* and *Firmicutes* interact with one another and contribute to obesity when the abundance ratios are out of balance. When this occurs, these bacteria are able to harvest excess energy from the host's diet. In the small intestine, aerobic and facultative anaerobes handle simple carbohydrates with ease in a rapid and efficient manner, while strict anaerobes colonize in the colon and digest fiber products that must experience fermentation before

their nutrients can be utilized (Flint et al., 2012). Understanding which bacteria are present in various locations in the GIT makes it possible to individualize medicine and prevent and/or treat diseases and metabolic disorders through nutritional intervention (Filippis et al., 2018). There is interest in how diets with varying carbohydrate sources alter alpha- and beta-diversity of the microbiome when there are varying concentrations of readily metabolizable carbohydrates compared to nondigestible carbohydrates included in the diet. Pigs can be used as a model to determine microbial differences due to their basic anatomical and physiological similarities to humans in digestion and absorption (Bassols et al., 2014).

Objective: To determine the effects of simple or complex carbohydrate sources on the gut microbiome and growth parameters of nursery piglets while characterizing the development and location of the microbiota.

MATERIALS AND METHODS

Animals

A total of 96 terminal cross-bred barrows and gilts were randomly assigned to 16 pens with 6 pigs/pen and BW of 5.5 ± 0.5 kg with equal representation of sex in each pen. The pigs were 21 d of age and the room holding the pigs was temperature controlled. The experiment took place over 28 days, pigs had ad libitum access to feed and water for the entire duration of the experiment, and sample collection of colonic and ileal digesta and mucosa took place immediately after pigs were placed in their respective pens from an extra 6 pigs on d 0 without being exposed to any of the nursery diets.

Diets and Collection Method

Body weight and feeder weights were measured on d 0, 7, 14, 21, and 28 to determine average daily feed intake (ADFI), average daily gain (ADG), and gain to feed (G:F). Ileal and colonic digesta and mucosa samples were taken on d 0, 14, and 28 from n = 6 on d 0 and n = 32 on d 14 and 28. The 4 diets were randomly assigned to 4 pens each and included Nutrisure™ (a food-grade cooked cereal grain product), oatmeal (food-grade oatmeal grain), steam-rolled oats (SRO), or dried-distillers grains as a negative control (DDGS). Diets comprised of similar inclusions of all ingredients within phase with 10% of each carbohydrate source, except for DDGS which was included at 15%, as shown in Table 1.1 and 1.2. Less Dairy-lac 80 was needed in the Nutri-Sure and Oatmeal diets because these ingredients already contained lactose and adjustments were made to include 8% lactose content in every diet. Indispensable amino acids were supplemented to meet NRC (2012) requirements for lysine, methionine, and threonine. Calcium was supplemented by inclusion of coarse ground limestone and phosphorus was supplemented evenly in the diets with the addition of dicalcium phosphate. In phase 2, fish meal was not included due to satisfaction of the crude protein content using only soybean meal and amino acid supplementation. All NRC (2012) requirements were met by nutrient inclusions in every diet and diet composition and calculated nutrient composition are illustrated in Tables 1.1-1.4.

DNA Isolation and Sequencing

A Mag Bind Soil DNA Kit (Omega Bio-tek, Norcross, GA), was used to extract DNA from luminal contents and tissue scrapings in both the ileum and the colon and

isolated in a KingFischer Isolation system (ThermoFisher Scientific, Waltham, MA). The DNA was amplified using polymerase chain reaction and profiled for quality and quantity with 1.5% DNA Gel Agarose Electrophoresis. Samples were then normalized using a Charm Biotech 96 well Normalization Kit and pooled together, all using an EpMotion Robot. Once pooled, the samples were purified using a NucleoSpin Gel and PCR Clean-Up kit (Takara Bio, Mountain View, CA) and quantified with a Denovix kit and reader (DeNovix, Wilmington, DE). Libraries were shown to be eligible for a 2 nM sequencing run using a V2 kit with an Illumina Next Generation Sequencer.

Statistical Analysis

DNA analysis began with the filtering of sequence reads as well as trimming to remove chimeras that could alter results. An ASV table was generated in R using Dada2 and a phylogenetic tree in Mothur. The tree was merged with the ASV table in R, a mapping file designed for variables of interest was added, and taxa was assigned from a Silva taxonomy package. Bray Curtis distances were generated to determine relative abundance measure in the MicrobiomeSeq and DESeq2 packages. Amplicon sequence variants (ASVs) were used as opposed to OTUs for more accurate analysis. The Adonis and Permanova functions included in the Vegan package were used to generate P-values to identify locational effects and which taxa were significantly different. RStudio was also used to generate principal component of analysis plots from Bray Curtis values as well as alpha diversity.

Growth performance analysis was determined with a 1-way analysis of variance (ANOVA) conducted using the MIXED procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC)

to differentiate treatment effects at a significance level of $P \leq 0.05$ and trends at a significance level of $0.05 < P < 0.15$.

RESULTS

Growth Performance

There were no effects of diet ($P > 0.10$) on ADFI, ADG, BW, or G:F overall. However, there was an increase ($P = 0.03$) in ADFI in wk 1 and 2 for consumption of SRO, NS, and OM diets compared to DDGS. As a result, diet produced an increase in ADFI in phase 1 ($P = 0.02$); however, by the time phase 2 began and for the overall experiment, there were no differences in growth performance among the diets. This is shown in Figures 1.4-1.7 where no differences are detectable.

Microbiome Analysis

There were no differences in overall microbial abundances or diversity among diets ($P = 0.189$), but a trend for time to affect microbial composition ($P = 0.111$). The location that samples were isolated from had varying microbial abundances ($P = 0.003$), leading to further analysis to determine which of the 4 locations were different. While it was determined that mucosa and digesta differ from each other ($P = 0.001$), they do not differ when extracted from the ileum versus the colon ($P > 0.10$). *Lachnospiraceae* *Pseudobutyrvibrio* and *Lachnospiraceae* *Butyrvibrio* were the top 2 families that caused differences and were most prominent in the mucosa compared to the digesta ($P < 0.01$). *Prevotellaceae* *Prevotella_1* was found in the colonic mucosa (CM) more than any other location ($P < 0.01$), *Ruminococcaceae* *Faecalibacterium* was of great abundance in the

ileal digesta (ID) ($P < 0.01$), and *Lachnospiraceae Lachnoclostridium* was the most present genus in the colonic digesta (CD) ($P < 0.01$) When comparing colonic digesta and mucosa, there was a greater abundance of *Helicobacteraceae Helicobacter*, *Campylobacteraceae Campylobacter*, *Prevotellaceae Prevotellaceae*, and *Rikenellaceae Rikenellaceae_RC9_gut_group* in the mucosa ($P < 0.01$) and *Erysipelotrichaceae Erysipelotrichaceae* and *Lactobacillaceae Lactobacillus* in the digesta ($P < 0.01$). The comparison between ileal mucosa (IM) and CD showed a greater abundance of *Ruminococcaceae Subdoligranulum*, *Prevotellaceae Prevotella*, *Muribaculaceae, Prevotellaceae Prevotellaceae_NK3B31_group*, *Veillonellaceae Megasphaera*, *Lachnospiraceae Coprococcus_3*, *Lachnospiraceae Dorea*, *Ruminococcaceae Ruminococcaceae*, *Veillonellaceae Dialister*, and *Streptococcaceae Streptococcus* in the colon ($P < 0.01$). The contrasts between ID and CM showed a greater abundance of *Campylobacteraceae Campylobacter*, *Muribaculaceae, Prevotellaceae Prevotellaceae_NK3B31_group*, *Prevotellaceae Prevotella_2*, *Rikenellaceae Rikenellaceae_RC9_gut_group* and *Enterobacteriaceae Escherichia/Shigella* in the CM ($P < 0.01$). The ileal components were compared and showed a greater abundance of *Veillonellaceae Dialister*, *Streptococcaceae Streptococcus*, *Lactobacillaceae Lactobacillus*, *Bifidobacteriaceae Bifidobacterium*, *Coriobacteriaceae Olsenella*, and *Clostridiaceae_1 Clostridium_sensu_stricto_1* in the ID ($P < 0.01$) while a great amount of unidentified bacteria were prominent in the IM ($P < 0.01$).

DISCUSSION

Growth performance was not affected by diet overall; however, growth performance was affected in weeks 1 and 2 and phase 1. The greater consumption of SRO, NS, and OM diets compared to the DDGS could be due to the sweeter smell of these diets that are higher in starch and more palatable (Seabolt et al., 2010). If the piglets are more likely to eat this diet in the first phase after weaning when stress is high, it's worth noting that if there's a supplement that requires enhanced feed intake, these diets would be better to feed than the DDGS. While there was a difference in feed intake, there were still no differences in ADG, G:F, or BW, meaning that even though the feed was more well liked, it was not more efficient and cost should be taken into consideration when feeding these diets if more product is used with less utilization in the body.

The gut microbiota composition and diversity were measured in 4 locations in the GIT including; luminal contents in the ileum and colon (ID, CD) and scrapings in the ileum and colon (IM, CM). Figure 1.3 shows the consistency in bacterial diversity among diets, but how composition changes in different sampling locations. There is also a presence of the *Planctomycetes* and *Cyanobacteria* phyla in this figure, bacteria that are found in chloroplasts and participate in photosynthesis. They are included in this study because when isolating DNA from the luminal contents, plant DNA residues were sequenced because they were extracted from the soybean meal and corn that the pigs ingested and were moving through the lumen at the time of sampling. The principal component analysis plot in Figure 1.2 shows the clustering of sample location, where clustering of one color represents that variable as being similar to itself and not to other locations. The abundance of *Lachnospiraceae* *Pseudobutyrvibrio* and *Lachnospiraceae*

Butyrivibrio were the most different among sampling location and these taxonomies are common to the gut and degrade complex polysaccharides to SCFA (Sagheedu et al., 2016). These families were harbored in the lining of the gut and protect against infections in the humans. It has been shown that low-level subtherapeutic antibiotics optimize *Lachnospiraceae* colonization in the infant mouse and contribute to the development in diabetes (Kameyama et al., 2014), most likely due to their energy precursor producing abilities.

In the CM, *Prevotellaceae Prevotella_1* genus was most abundant and secretes ghrelin, a gut hormone that maintains dopamine function and upholds GIT motility (Gerhardt et al., 2018). *Campylobacteraceae Campylobacter* colonized in the CM, as well and is found in house flies and it has been previously shown that a majority of *C. Campylobacter* contain virulence genes and antibiotic resistance (Scanlon et al., 2013). This species is closely related to *Muribaculaceae* that was also found in the CM as a result of the ingestion of flies by the pigs. As far as public health concern goes, this discovery should not be of concern if pork is properly cooked and this genus could be a target for research to better understand the mechanisms of antibiotic resistance.

Helicobacteraceae Helicobacter is a known agent of gastrointestinal disease; however, some species are not pathogenic (Oxley et al., 2004). *Rikenellaceae*

Rikenellaceae_RC9_gut_group had high presence in the CM and participates in lipid metabolism, commonly leading to obesity and diabetes (Cani et al., 2013). The bacteria present in the CM, the most diverse location in the GIT, showed many beneficial bacteria that participated in membrane integrity, while there were species that are pathogenic.

This could suggest that there are parts of the GIT that are more prone to infection than others and the source of stress is identifiable based on the type of infection.

In the CD, the abundant *Ruminococcaceae Subdoligranulum* and *Ruminococcaceae Ruminococcaceae* have been proven to affect gut barrier function and participate in colonization resistance against *Clostridium difficile*, a bacterium associated with antibiotic-associated diarrhea (Pérez-Cobas et al., 2014). *Veillonellaceae Megasphaera* are a commensal bacterium with a genome involved in metabolism of proteins and amino acids as well as carbohydrates that produce end products beneficial to the host, such as SCFA, vitamins, and co-factors for other necessary cell functions (Shetty et al., 2013). *Lachnospiraceae Coprococcus_3* is a known butyrate producer, one of the major sources of energy to the colonic mucosa cells that also participates in regulating gene expression, inflammation, differentiation, and apoptosis (Noriega et al., 2016). The two *Prevotellaceae* groups, *Prevotellaceae Prevotella* and *Prevotellaceae Prevotellaceae_NK3B31_group* were among the top 10 families most abundant in the CD, which could be the result of sloughing of the intestinal lining, as these genus's are commonly found in the mucosa. The *Lachnospiraceae Dorea* is a major gas producer in the GIT with glucose fermentation end products including hydrogen and carbon dioxide (Taras et al., 2002). *Veillonellaceae Dialister* has been shown to increase the amount of circulating IL-6 in the blood serum, a cytokine that communicates with the host to stimulate the immune system (Martinez et al., 2012). Lastly, *Streptococcaceae Streptococcus* was abundant in the CM and is known to cause infections, such as pharyngitis and impetigo (Walker et al., 2014). The presence of both the beneficial and degenerative bacteria agrees with the findings in the CM that pathogenic bacteria may

reside in a specific location in the GIT and medicine may be targeted towards those areas to prevent an imbalance in earlier sections of the intestine from the effects of antibiotics.

The digesta samples were not as diverse as the mucosa samples; however, there were certain genus' that appeared more often in the digesta than the mucosa.

Veillonellaceae Dialister and *Streptococcaceae Streptococcus* were shown to be in a high abundance in the luminal contents more than in the lining. *Lactobacillaceae*

Lactobacillus were also copious and is commonly found in probiotics as this genus is involved in carbohydrate metabolism to produce lactic acid (Walter et al., 2008).

Bifidobacteriaceae Bifidobacterium are among the first bacteria that colonize in the GIT and are also probiotic organisms that perform health-promoting actions and also

participate in carbohydrate metabolism (Egan et al., 2018). The *Coriobacteriaceae*

Olsenella genus is present in both healthy and acidotic animals and is involved in carbohydrate metabolism, producing lactic acid in ruminants and non-ruminants from various carbohydrate sources (Kraatz et al., 2010). The *Firmicutes* family in the ileal bacteria community was composed of a high presence of *Clostridiaceae_1*

Clostridium_sensu_stricto_1 for protein and amino acid metabolism and utilization (Fan et al., 2017); however, it has been shown that a decrease in dietary protein results in an increase in this strain of bacteria and improvement of ileal barrier integrity. The digesta is continuously moving through the GIT and is subjected to many more microbes than the stationary mucosa; however, it maintains less diversity.

CONCLUSION

Growth performance and the overall gut microbiome were not affected by time or diet; however, there were differences in microbial composition when comparing the luminal contents and lining. These observations suggest that sample extraction from the colon may be representative for bacteria present in the ileum if the microbes isolated are from the mucosa or the digesta, but not intermixed. Energy producing bacteria were also found in both the ileum and the colon, showing that microbes in the latter portion of the intestine do not rely on the energy produced in the former. Complex carbohydrates that are broken down during fermentation are useful to supplement these bacteria to keep communities diverse and efficient. The *Lachnospiraceae* family of the *Clostridiales* order is the main cause for variation between digesta and mucosa while originally assumed to have been most prominent in the mucosa. This observation agrees with previous studies that have shown a greater abundance in the mucosal lining that affects gut integrity and energy production. There are many species that differ from sampling location, both beneficial and pathogenic, suggesting that phenotype may be influenced by supplementation that targets specific sections of the GIT. It is of interest to determine if treatment of one part of the intestine without disrupting the microbiome in other parts can improve immune status of a sick individual through preservation of the unaffected microorganisms that produce health-enhancing co-products. Further characterization of the microbiome and the production of metabolites may help provide opportunity for specific outcomes through nutritional intervention.

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TABLES

Table 1.1. Ingredient composition (% as-fed bases) of diets for Phase 1.

Ingredients, %	DDGS	SRO	NS	OM
Corn	32.6	36.5	39.1	40.3
Soybean meal, Dehulled, Solvent Extracted	32.0	33.0	33.0	33.0
Fish Meal	7.00	7.00	7.00	7.00
Corn DDGS, > 6% and < 9% oil	15.0	0.00	0.00	0.00
Steam-Rolled Oats	0.00	10.0	0.00	0.00
Nutrisure	0.00	0.00	10.0	0.00
Oatmeal	0.00	0.00	0.00	10.0
Dairylac-80	8.00	8.00	5.40	4.20
Corn Oil	3.00	3.00	3.00	3.00
Dicalcium Phosphate 18.5% P	0.40	0.65	0.65	0.70
Coarse Ground Limestone	0.85	0.70	0.65	0.62
L-Lys-HCl	0.32	0.32	0.34	0.34
DL-Met	0.04	0.04	0.06	0.06
L-Thr	0.06	0.10	0.12	0.12
Sodium Chloride	0.30	0.30	0.30	0.30
Vitamin Premix¹	0.25	0.25	0.25	0.25
Trace Mineral Premix²	0.15	0.15	0.15	0.15

¹Vitamin premix supplied per kg of diet: vitamin A (as retinyl acetate), 5500 IU; vitamin D (as cholecalciferol), 550 IU; vitamin E (as tocopherol acetate), 30 IU; vitamin K (as menadione dimethylpyrimidinol bisulfate), 4.4 mg; riboflavin, 11.0 mg; d-pantothenic acid, 22.05 mg; niacin, 33.0 mg; vitamin B₁₂ (as cyanobalamin), 33.0 mg.

²Trace mineral premix: copper (as CuSO₄·5H₂O), 10 mg/kg; iodine (as Ca(IO₃) · H₂O), 0.25 mg/kg; iron (as FeSO₄ · 2H₂O), 125 mg/kg; manganese (MnO), 15 mg/kg; selenium (Na₂SeO₃), 0.3 mg/kg; zinc (ZnSO₄ · H₂O), 125 mg/kg.

Table 1.2. Calculated nutrient composition of diets for Phase 1.

Calculated nutrient composition, %	DDGS	SRO	NS	OM
ME ¹ , kcal/kg	3446	3357	3499	3502
Crude protein	27.1	25.0	24.8	25.0
SID² AA, %				
Arg	1.52	1.48	1.46	1.46
His	0.60	0.56	0.56	0.56
Ile	0.95	0.89	0.89	0.89
Leu	1.96	1.68	1.71	1.72
Lys	1.51	1.50	1.50	1.50
Met	0.45	0.45	0.43	0.43
Met+Cys	0.77	0.76	0.73	0.73
Phe	1.09	1.00	1.00	1.00
Phe+Tyr	1.85	1.67	1.66	1.66
Thr	0.89	0.88	0.89	0.89
Trp	0.26	0.26	0.25	0.25
Val	1.06	0.98	0.97	0.97
STTD ³ Phosphorus	0.45	0.45	0.44	0.45
Total Phosphorus	0.71	0.72	0.72	0.72
Calcium	0.85	0.85	0.85	0.85
Fermentable fiber	14.7	12.0	11.2	11.3
NDF	10.1	7.14	6.27	6.38
Starch	22.5	27.5	30.9	30.6
Sugar	7.81	7.85	7.87	7.92

¹ME: metabolizable energy

²STTD: standardized total tract digestible; SID: standard ileal digestibility

Table 1.3. Ingredient composition (% as-fed bases) of diets for Phase 2.

Ingredients, %	DDGS	SRO	NS	OM
Corn	36.8	40.7	43.3	44.5
Soybean meal, Dehulled, Solvent Extracted	38.0	39.0	39.0	39.0
Fish Meal	0.00	0.00	0.00	0.00
Corn DDGS, > 6% and < 9% oil	15.0	0.00	0.00	0.00
Steam-Rolled Oats	0.00	10.0	0.00	0.00
Nutrisure	0.00	0.00	10.0	0.00
Oatmeal	0.00	0.00	0.00	10.0
Dairylac-80	4.00	4.00	1.40	0.14
Corn Oil	3.00	3.00	3.00	3.00
Dicalcium Phosphate	1.10	1.35	1.40	1.40
Coarse Ground Limestone	1.05	0.90	0.80	0.85
L-Lys-HCl	0.26	0.28	0.30	0.30
DL-Met	0.04	0.04	0.08	0.08
L-Thr	0.00	0.06	0.06	0.06
Sodium Chloride	0.30	0.30	0.30	0.30
Vitamin Premix¹	0.25	0.25	0.25	0.25
Trace Mineral Premix²	0.15	0.15	0.15	0.15

¹Vitamin premix supplied per kg of diet: vitamin A (as retinyl acetate), 5500 IU; vitamin D (as cholecalciferol), 550 IU; vitamin E (as tocopherol acetate), 30 IU; vitamin K (as menadione dimethylpyrimidinol bisulfate), 4.4 mg; riboflavin, 11.0 mg; d-pantothenic acid, 22.05 mg; niacin, 33.0 mg; vitamin B₁₂ (as cyanobalamin), 33.0 mg.

²Trace mineral premix: copper (as CuSO₄·5H₂O), 10 mg/kg; iodine (as Ca(IO₃) · H₂O), 0.25 mg/kg; iron (as FeSO₄ · 2H₂O), 125 mg/kg; manganese (MnO), 15 mg/kg; selenium (Na₂SeO₃), 0.3 mg/kg; zinc (ZnSO₄ · H₂O), 125 mg/kg.

Table 1.4. Calculated nutrient composition of diets for Phase 2.

Calculated nutrient composition, %	DDGS	SRO	NS	OM
ME ¹ , kcal/kg	3446	3357	3499	3502
CP	27.1	25.0	24.8	25.0
SID² AA, %				
Arg	1.52	1.48	1.46	1.46
His	0.60	0.56	0.56	0.56
Ile	0.95	0.89	0.89	0.89
Leu	1.96	1.68	1.71	1.72
Lys	1.51	1.50	1.50	1.50
Met	0.45	0.45	0.43	0.43
Met+Cys	0.77	0.76	0.73	0.73
Phe	1.09	1.00	1.00	1.00
Phe+Tyr	1.85	1.67	1.66	1.66
Thr	0.89	0.88	0.89	0.89
Trp	0.26	0.26	0.25	0.25
Val	1.06	0.98	0.97	0.97
STTD ² Phosphorus	0.45	0.45	0.44	0.45
Total Phosphorus	0.71	0.72	0.72	0.72
Calcium	0.85	0.85	0.85	0.85
Fermentable fiber	14.7	12.0	11.2	11.3
NDF	10.1	7.14	6.27	6.38
Starch	22.6	27.5	30.9	30.6
Sugar	7.81	7.85	7.87	7.92

¹ME: metabolizable energy

²STTD: standardized total tract digestible; SID: standard ileal digestibility

FIGURES

Figure 1.1. Alpha Diversity measures of location samples were extracted (Sampletype) compared among diets.

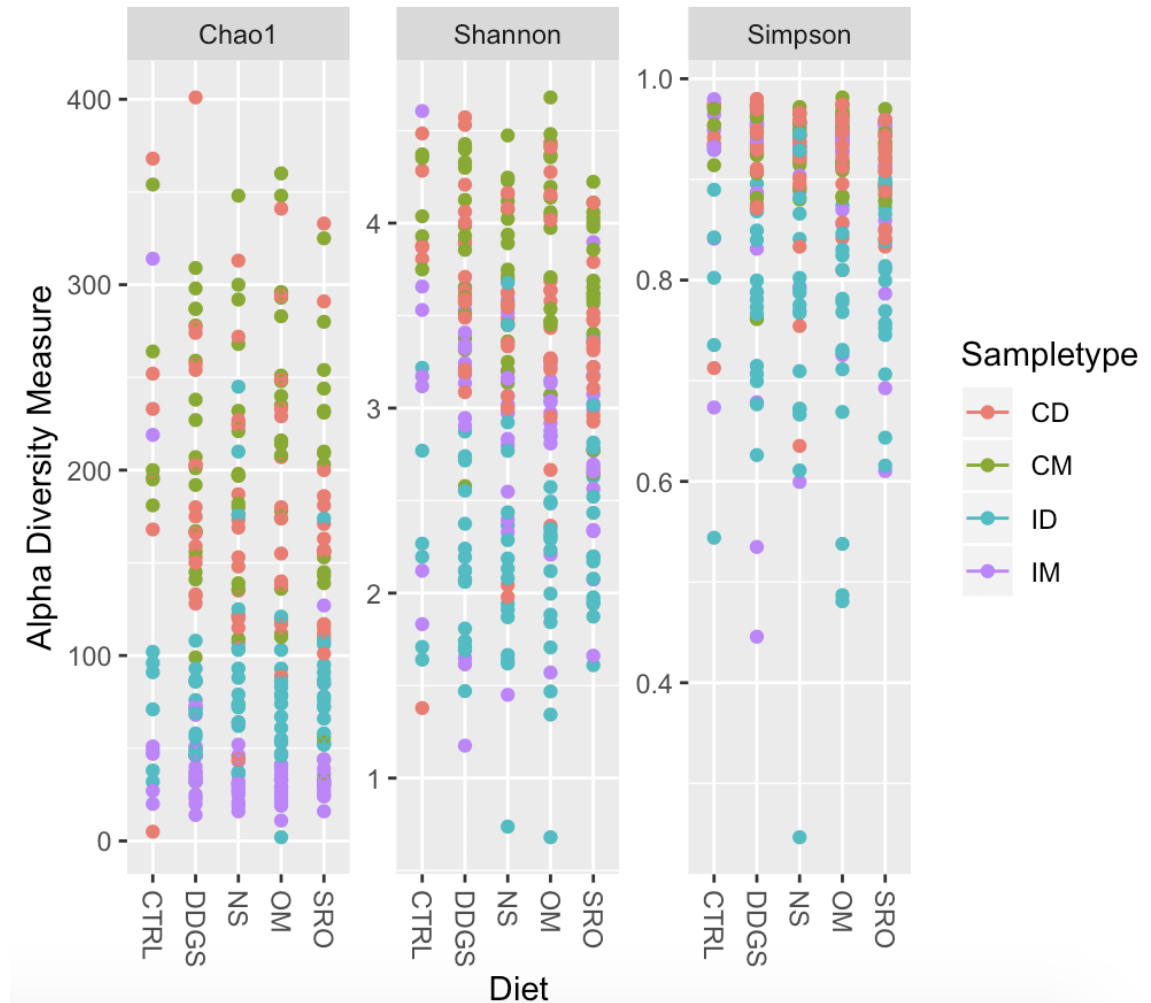


Figure 1.2. Relative abundance principal component of analysis plot with variables sample type versus diet.

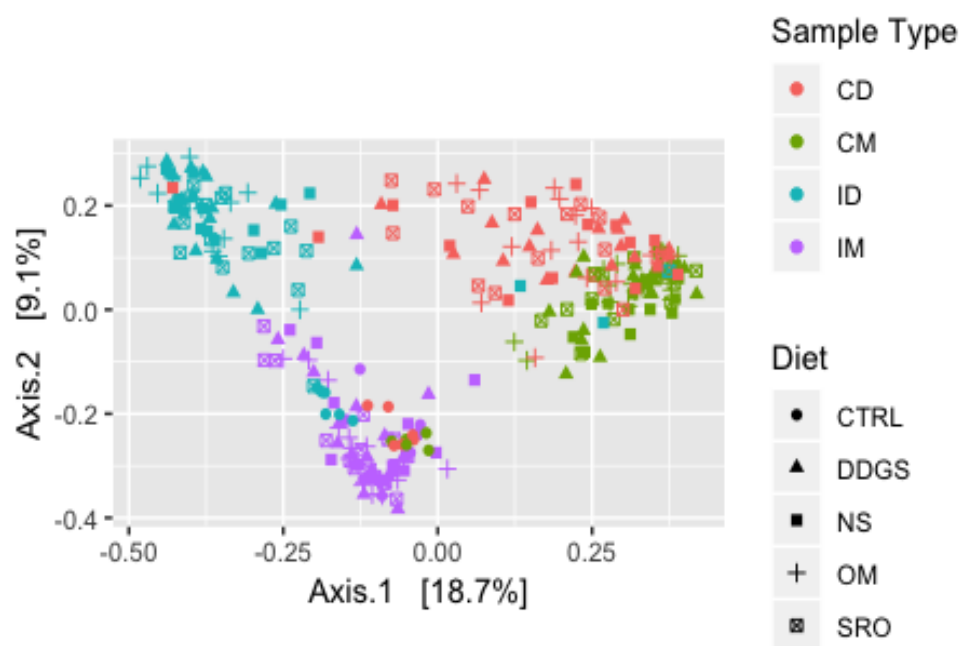


Figure 1.3. Phylum composition of sampling site bacterial communities by diet for ID, CD, IM, and CM.

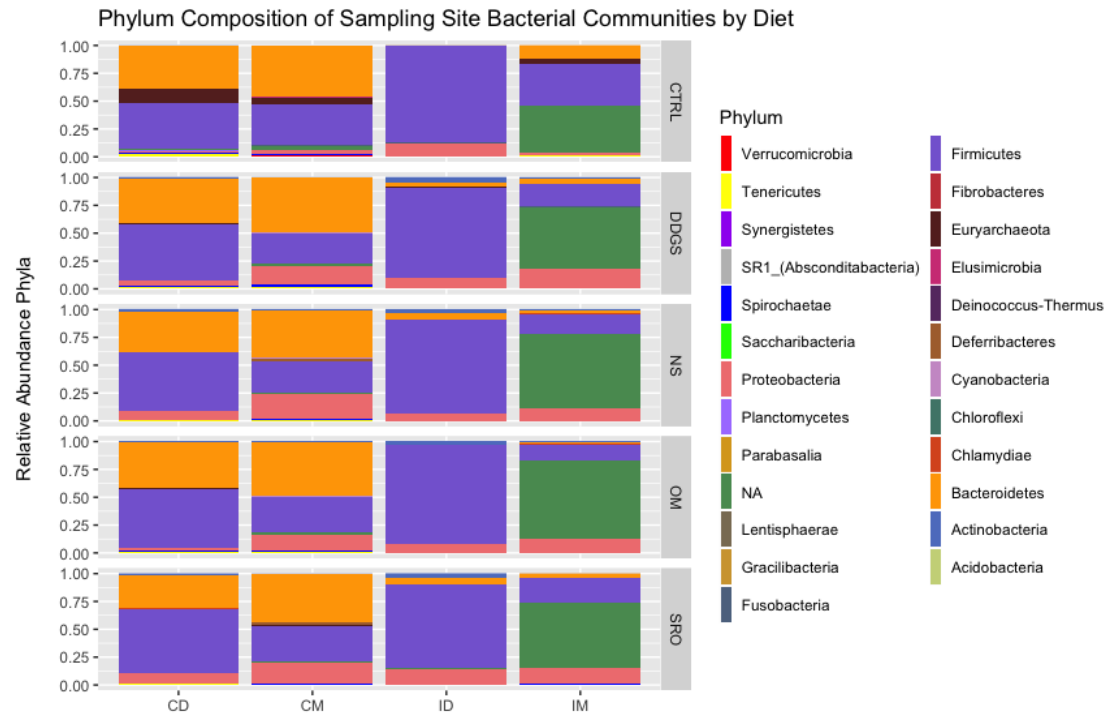


Figure 1.4. Average daily gain within diet in wk 1-4.

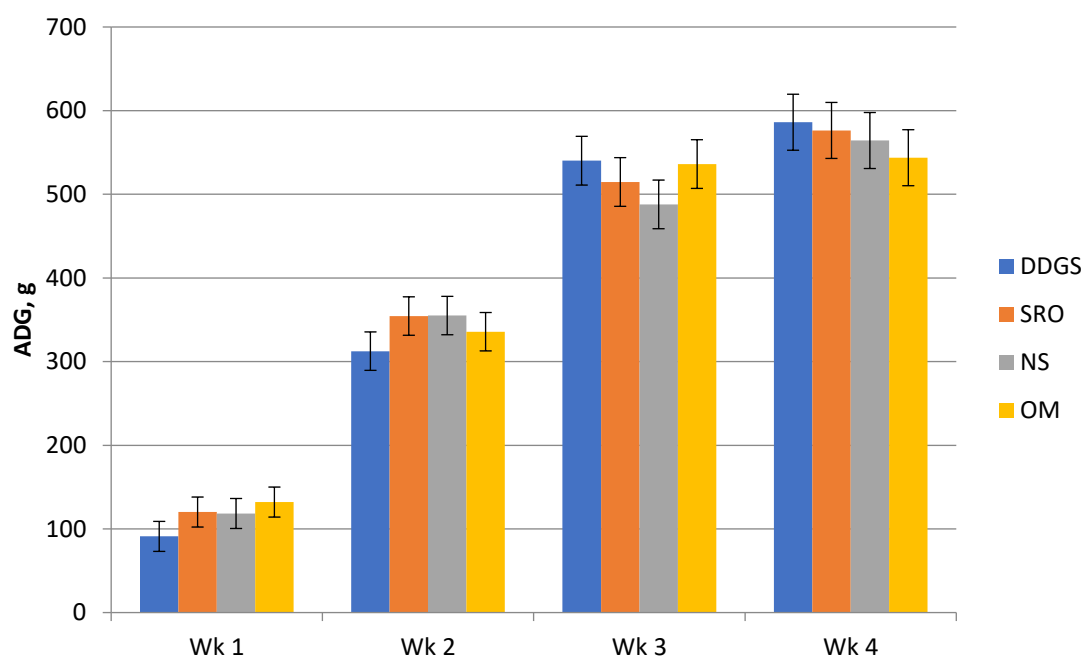


Figure 1.5. Average daily feed intake within diet in wk 1-4.

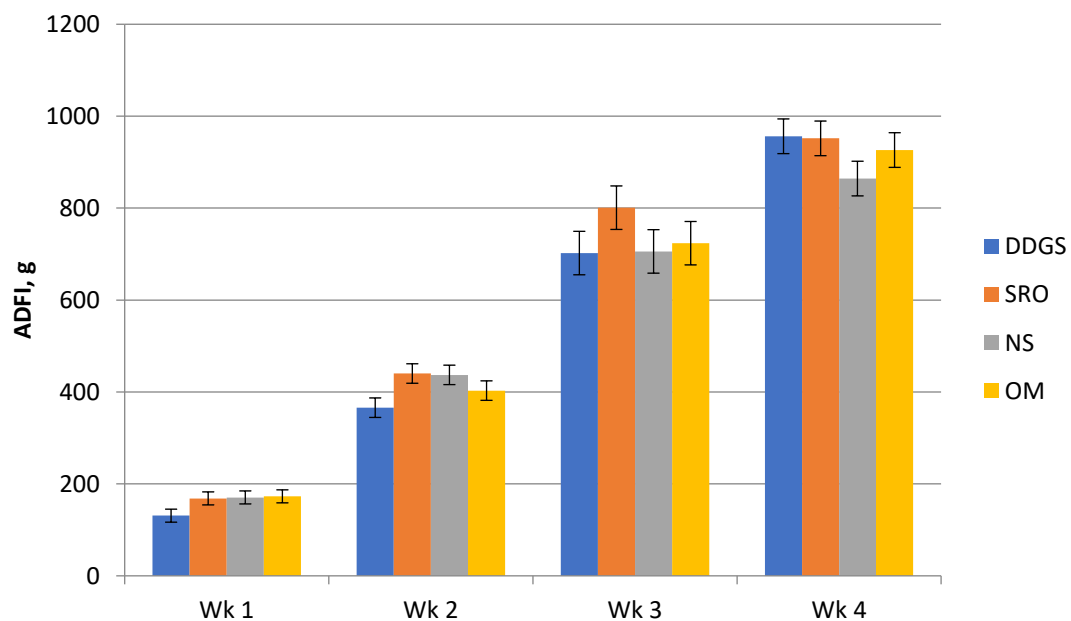


Figure 1.6. Gain:feed within diet in wk 1-4.

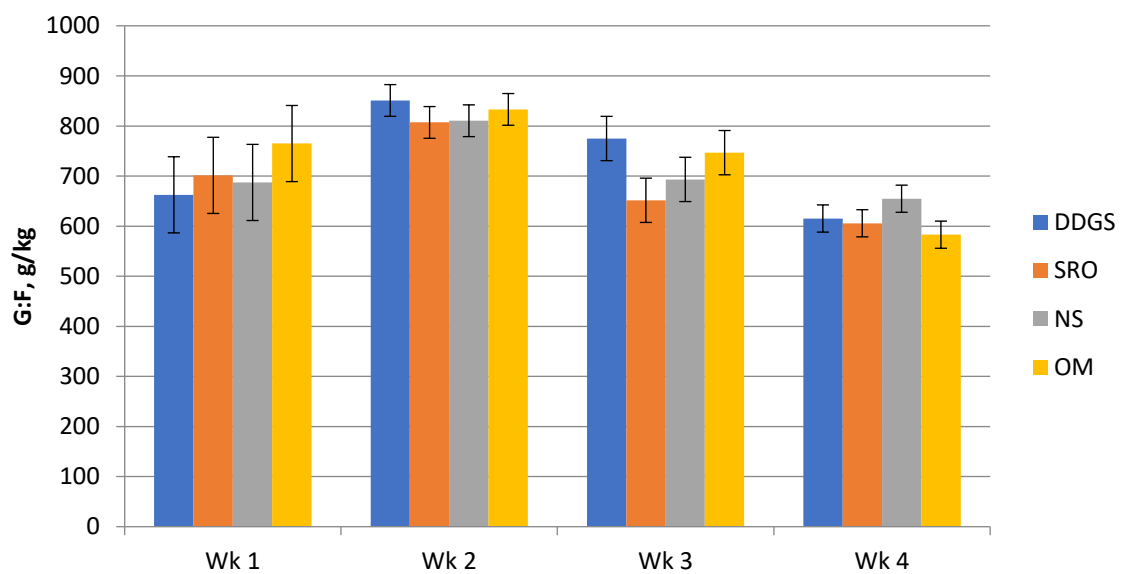
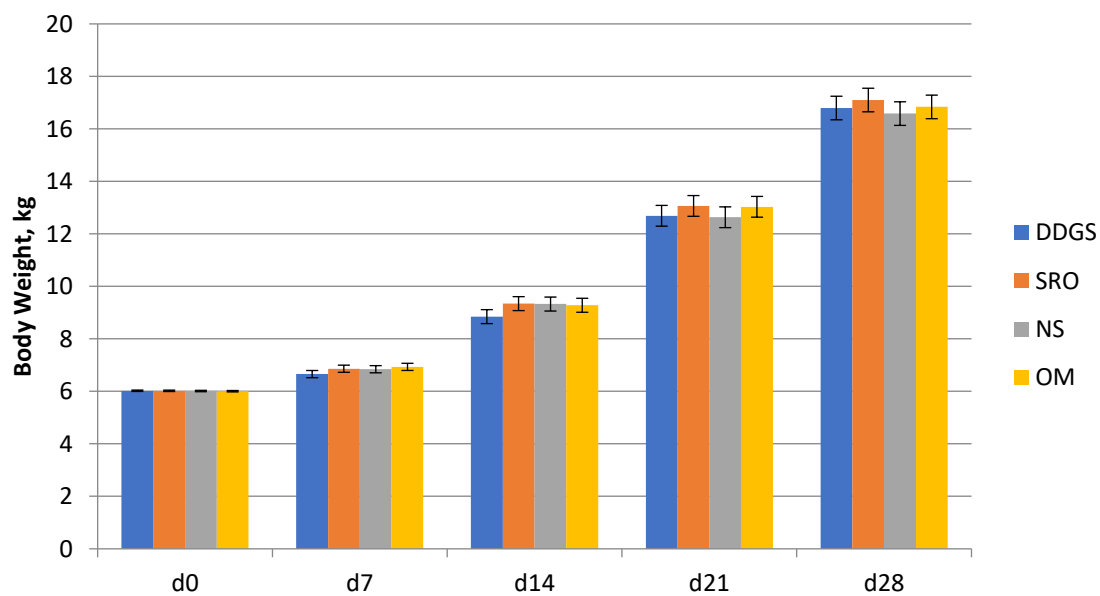


Figure 1.7. Body weight within diet on d 0, 7, 14, 21, and 28.



**Chapter 3: Effects of soybean-derived isoflavones on nursery pig growth
performance, microbiome, and immune parameters.**

ABSTRACT

The effects of soybean-derived isoflavones on the microbiome, growth performance, and interleukin-8 (IL-8) were observed in nursery pigs. Sixty-four pigs weaned at 21 d were randomly allotted to 16 pens (4 pigs/pen; 4 pens/treatment) with sex represented evenly in each pen. Pigs had ad libitum access to feed and water for the 2-phase nursery period (28 d). Dietary treatments included: 1) Hamlet protein's HP-300 (highly digestible soybean meal product), 2) HP-300 + Novasoy 400 (isoflavone supplement), 3) Arcon-F (soy protein concentrate in place of soybean meal), and 4) Arcon-F + Novasoy 400. Fecal samples were collected on d 0, 14, and 28 ($n = 32$) as well as blood samples for IL-8 enzyme-linked immunofluorescence assay (ELISA) while ileal and colonic mucosa and digesta samples were collected solely on d 28 ($n = 16$) for analysis of microbial species using IlluminaTM next generation sequencing. The DNA sequences were filtered through the DADA2 pipeline, Mothur, and QIIME. Pig body weights and feeder weights were also measured on d 0, 7, 14, 21, and 28 ($n = 64$) for growth performance analysis. There were no differences in growth performance for body weight (BW), average daily feed intake (ADFI), average daily gain (ADG), or gain:feed (G:F); ($P > 0.10$). While diet did not affect the amount of circulating IL-8, there was an increase in the concentration of blood serum IL-8 over time ($P < 0.001$). Diet, day, and sample location caused differences in microbial composition ($P < 0.01$) with most variation resulting from the Arcon-F diet that had a lower presence of the *Chlamydiae*, *Fusobacteria*, and *Proteobacteria* phylums. The composition of bacteria varied over time with an increase in *Chlamydiae*, *Vertebrata*, *Cyanobacteria*, and *Spirochaetes* by d 14 ($P < 0.001$), but these differences were not detected between d 14 and 28. Colonic digesta

was the least diverse with a decrease in many phyla, most notably *Bacteroidetes*, *Firmicutes*, *Cyanobacteria*, and *Actinobacteria* ($P < 0.005$).

Key words: gastrointestinal microbiota, immunity, isoflavones, nursery piglets

INTRODUCTION

With the introduction of the Veterinary Feed Directive and consumer demands, most producers are moving towards using feed ingredients that improve gut health with nutritional intervention in production to amplify the immune system and decrease the use of antibiotics. A member of the legume family, soybeans are inexpensive and easily processed to a defatted version that is popular to consume for both humans and animals. In swine, soybean meal is a common ingredient used for its high protein content and digestibility with over 4.06 billion bushels of soybeans produced in the U.S. in 2016 alone, with numbers increasing annually (Schulz et al., 2016). Once the hulls are removed, the soybean oil is extracted and the solvent is removed, the leftover flakes are a product high in quality protein content with most anti-nutritional factors removed (Johnson et al., 1995). In processed soybean meal, the phytoestrogens classified as isoflavones remain intact and retain bioactivity to influence many immune parameters of interest. Isoflavones are ingested mainly in their glycosidic form (daidzin, genistin, and glycitin) before β -glycosidases that reside in bacteria and the epithelial lining of the host cleave the glycosidic groups to their aglycosidic form (daidzein, genistein, and glycitein); (Aglin et al., 2013). Resembling estrogen, isoflavones exert either weak estrogenic or antagonistic effects conditionally, depending on the age, health status, sex, and the composition of the gut microbiome in the host. Genistein and daidzein are the bioactive forms formed during digestion from the gut microbiota and cells from zymogens genistin and daidzin; however, there is interest in the metabolite equol as it may have enhanced estrogenic activity than these two compounds combined (Anupongsanugool et al., 2005). Isoflavones are of interest due to their anti-inflammatory, antiviral, and antimicrobial

properties as well as in some cases, relieving cancer patients; however, adverse effects in cancer patients suggest that there are appropriate situations to use the compounds, depending on if the patient has more estrogen receptor (ER) α or β (Russo et al., 2016). There is interest to understand which gut microbes are able to digest the isoflavones and produce the beneficial metabolites that induce the immune system to increase circulating cytokines, maintain a diverse and healthy gut microbiome to combat disease when introduced, maintain intestinal epithelial integrity, and positively effect growth performance.

Objective: To determine the effects of isoflavones derived from a soybean meal product compared to a soy protein concentrate with low anti-nutritional factors and an isoflavone concentrate added to both products (Arcon-F, Arcon-F + Novasoy 400, HP-300, HP-300 + Novasoy 400) on nursery pig growth, feed efficiency, gastrointestinal microbiota, and interleukin-8.

MATERIALS AND METHODS

Animals

Piglets (n = 64; WXL/DNA Genetics Landrace/DNA Genetics Yorkshire \times DNA Genetics Duroc) were weaned at 21 d of age and allotted to 16 experimental pens with equal representation of sex in each pen (n = 4, males = 2, females = 2), similar body weight (BW) of 5.5 ± 0.5 kg, and nearly even distribution of litters. Pigs were given ad libitum access to feed and water throughout the experiment. All lights in the experimental room remained on 24 h/d throughout the duration of the experiment. Room temperature was 28° C during wk 1 and dropped by 2 degrees each wk, thereafter heat lamps were

used during the first 2 wk to provide extra heat inside the pens. The entire experiment lasted for an interval of 28 d and sample collection of feces occurred after pigs were positioned in their respective pens on d 0, 14, and 28 from n = 32 pigs and diets were randomly assigned. Ileal mucosa (IM), ileal digesta (ID), colonic mucosa (CM), and colonic digesta (CD) samples were also obtained on d 28 from n = 16 pigs. Body weight and feeder weights were recorded on d 0, 7, 14, 21, and 28 to estimate average daily feed intake (ADFI), average daily gain (ADG), and gain:feed (G:F).

Diets

Four dietary treatments were fed in 2 phases and all diets were similar to a corn-soybean meal diet formulated to meet nursery pig requirements based on weight (NRC, 2012). The high bioavailable protein soybean product HP-300 (Hamlet Protein; Findlay, OH) and Arcon-F, a soy protein concentrate (ADM; Chicago, IL) were used to replace SBM and are referred to as HP-300 and Arcon-F diets, respectively. Diets were also formulated with these products combined with Novasoy 400 (ADM; Chicago, IL) to add supplemental isoflavones. The 2 diets with extra isoflavones were formulated to contain similar inclusions of isoflavones at 2,011 mg/kg and are referred to as HP+NS (HP-300 + Novasoy 400) or AF+NS (Arcon-F + Novasoy 400). These inclusions were determined by the concentration of isoflavones in a typical soybean meal diet and referencing literature that has included isoflavones up to 2,500 mg/kg in the diet while accounting for a margin of safety to prevent the deleterious effects. Similar nutrient content was maintained among diets within phase with no antibiotics or ZnO. Each diet phase was fed

for 2 wk and ingredient inclusion and calculated nutrient composition for each diet and phase is illustrated in Tables 2.1-2.4.

Data Collection

Feed disappearance and individual pig BW were measured weekly for 4 consecutive weeks post-weaning for the determination of average daily gain (ADG), average daily feed intake (ADFI), and gain:feed (G:F). Fecal and blood samples were collected on d 0, 14, and 28 from 2 pigs in each pen. Blood samples were analyzed for IL-8 concentrations in the serum and feces were used for characterization of the microbiome. Pigs (n = 16) were euthanized at the end of the study on d 28 and mucosal scrapings, and luminal contents from both the ileum and colon were collected for DNA analysis and microbiome composition determination.

DNA Isolation and Sequencing

A Mag Bind Soil DNA Kit (Omega Bio-tek, Norcross, GA), was used to extract DNA from luminal contents and tissue scrapings in both the ileum and the colon as well as from fecal samples. The obtained DNA was isolated in a KingFischer Isolation system (ThermoFisher Scientific, Waltham, MA) and amplified using polymerase chain reaction and profiled for quality and quantity with 1.5% DNA Gel Agarose Electrophoresis. Samples were then normalized and purified using an NGS Normalization 96-well kit (Norgen Biotek Corp, Thorold, Ontario) and pooled together. Samples were quantified with a Denovix kit and reader (DeNovix, Wilmington, DE) and purity assessed with a High Sensitivity DNA kit, chip, and Bioanalyzer (Agilent, Santa Clara, CA) to ensure

dimers were removed and peak concentrations were high enough. Libraries were found to be eligible for a 2 nM sequencing run using a V2 kit with a Next Generation Sequencer (Illumina, San Diego, CA).

IL-8 ELISA

A DuoSet ELISA Porcine IL-8/CXCL8 kit was used to measure porcine Interleukin 8 in serum isolated from blood. All reagents were brought to room temperature before use and components were allowed to sit for a minimum of 15 minutes with gentle agitation after initial reconstitution. Working dilutions were prepared and used immediately, except for the Wash Buffer and Reagent Diluent. The Capture Antibody was diluted to working concentration in PBS without the carrier protein and immediately used to coat a 96-well microplate, sealed, and incubated overnight at room temperature. The next day, wells were aspirated and washed with Wash Buffer 3 times using a manifold dispenser. The plate was then blocked with Reagent Diluent and incubated at room temperature for at least 1 hour and washed again. The samples were then added to the wells in duplicate to enhance reproducibility, covered with an adhesive strip, and incubated at room temperature for 2 hours. The plate was then washed before the Detection Antibody, diluted in Reagent Diluent and Normal Goat Serum, was added to each well, covered with a new adhesive strip, and incubated for 2 hours at room temperature. Samples were then washed before a working dilution of Streptavidin-HRP was added to each well and incubated for 20 minutes at room temperature while covered and out of direct light. The wash step occurred once more before the Substrate Solution was added to each well incubated for 20 minutes at room temperature out of direct light.

The Stop Solution was then added to each well and the plate was gently tapped to ensure thorough mixing before the optical density of each well was determined. The microplate reader was set to 450 nm and wavelength correction at 540 nm.

Statistical Analysis

The DNA analysis began with the filtering of sequence reads as well as trimming to remove chimeras that may skew results. An ASV table was generated in R using Dada2 and a phylogenetic tree in mothur. The tree was merged with the ASV table in R, a mapping file designed for variables of interest was added, and taxa were assigned from a Silva taxonomy package. Bray Curtis distances were generated to determine relative abundance measures in the MicrobiomeSeq and DESeq2 packages. Amplicon sequence variants (ASVs) were used as opposed to OTUs for more accurate analysis. The Adonis and Permanova functions included in the Vegan package were used to generate p-values to identify locational effects and which taxa were significantly different. R was also used to generate principal component of analysis plots from Bray Curtis values as well as alpha diversity.

Growth performance analysis was performed using a 1-way analysis of variance (ANOVA) conducted using the MIXED procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC) to differentiate treatment effects at a significance level of $P \leq 0.05$ and trends at a significance level of $0.05 < P < 0.15$.

RESULTS

Growth performance

Diet did not effect ADG, ADFI, BW, or G:F ($P > 0.10$). Body weight averaged 15.20 kg by day 28 with an ADG of 1,276.8 g. There was a decrease in feed intake for all pigs receiving diets during wk 4, with wk 3 averaging 1,170 g per diet per day while ADFI in wk 4 was only 793 g/d. No differences were observed at specific weeks or phases ($P > 0.10$); however, there was a trend for G:F to be greater in the Arcon-F + Novasoy 400 diet during wk 1 and 3 ($P = 0.113$).

Microbiome analysis

Significant differences in microbial composition and diversity were observed among diets, the location the samples were isolated from, and over time. The diets that produced significantly different microbial composition were the Arcon-F diet that maintained less diversity while the Arcon-F + Novasoy 400 saw an increase in abundance of genus'. The AF diet showed an increased abundance of the phyla *Chlamydiae*, *Fusobacteria*, and *Proteobacteria* ($P < 0.005$; Table 2.8). Genus' that were less abundant were *Veillonella*, *Lactobacillus*, *Christensenella*, *Lachnospiraceae*, *Chlamydia*, *Actinobacillus*, *Sphingomonas*, and *Bifidobacterium* ($P < 0.001$). At the phylum level, AFNS showed an increase in *Ruminococcaceae*, *Syntrophococcus*, *Allisonella*, *Schwartzia*, *Sanguibacteroides*, *Leptotrichia*, *Slackia*, *Collinsella*, *Olsenella*, *Enterococcus*, *Truicibacter*, and *Shuttleworthia* ($P < 0.001$)..

The microbiome became increasingly diverse over time ($P = 0.001$) as shown in Figure 2.1. After d 0, there was an increase in several genus' including *Euryarchaeota*,

Verrucomicrobia, *Planctomycetes*, *Synergistetes*, *Actinobacteria*, and *Lentisphaerae* ($P < 0.001$). By d 14, there was a greater abundance of *Chlamydiae*, *Vertebrata*, *Cyanobacteria*, *Fibrobacteres*, *Spirochates*, *Epsilonbacteraeota*, *Deferribacteres*, *Tenericutes*, and *Patescibacteria* ($P < 0.001$).

Microbial diversity varied between sampling location ($P = 0.001$) with most of the variation occurring in the CD; however, there was variation in the CM, as well. There was a decrease in abundance of *Blautia*, *Marvinbryantia*, *Fusicatenibacter*, *Dorea*, *Lachnospiraceae*, *Oribacterium*, *Dialister*, *Allisonella*, *Megasphaera*, *Mitsuokella*, *Selenomonas*, *Acidaminococcus*, *Phascolarctobacterium*, *Oscillibacter*, *Anaerofilum*, *Angelakisella*, *Faecalibacterium*, *Subdoligranulum*, *Pygmaibacter*, and *Butyricicoccus* in the CD ($P < 0.001$). The CM showed an upregulation of *Vibrionimonas*, *Sediminibacterium*, *Chlamydia*, *Klebsiella*, *Escherichia Shigella*, *Mycoplasma*, *Pelomonas*, *Pseudomonas*, *Stenotrophomonas*, *Bradyrhizobium*, *Methylobacterium*, *Helicobacter*, and *Bacillus* ($P < 0.001$). Microbial composition of the feces was not different from the CD ($P > 0.05$).

The top 10 families responsible for diversity differences included *Ruminococcaceae*, *Lachnospiraceae*, *Shuttleworthia*, *Allisonella*, *Slackia*, *Angelakisella*, *Turicibacter*, *Clostridium*, *Veillonella*, and *Negativabacillus* in decreasing order ($P < 0.001$).

IL-8 ELISA

The IL-8 concentration in blood serum did not change with diet ($P > 0.10$); however, there was an increase in circulating IL-8 over time ($P < 0.001$), except for in the

Arcon-F diet. This diet showed a decrease in IL-8 in the blood serum by d 28 as shown in Figure 2.7.

DISCUSSION

No differences were observed among diets for growth performance as ADG, ADFI, G:F, and BW remained consistent within phase. While there is always an interest in using products in diets that cause pigs to perform better than others, growth performance in this study cannot be included as a reason for microbiome diversity changes and the differences in observations can be reserved strictly for the variables of interest, diet, time, and sampling location.

The differences observed in microbial composition caused by diet were primarily in the diets containing the Arcon-F product. The AF diet showed less abundance of many bacteria that are typically high in the piglet microbiome. Of these, *Veillonella* is an early colonizer that forms a mutually beneficial relationship with the host through its role in lactic acid metabolism (Periasamy et al., 2010). *Lactobacillus* is also a beneficial bacterium that was found in lower abundance, putting these animals at a disadvantage in not being able to utilize their health-promoting properties, even though this species does not typically colonize in the gut, but is allochthonous (Walter et al., 2008).

Lachnospiraceae also colonize early and participate in carbohydrate metabolism to produce SCFA, energy sources for the microbiome (Sagheddu et al., 2016). *Schwartzia* is another phylum that produces energy for the GIT bacteria using succinate, a common metabolite in cellular processes, such as the citric acid cycle (Gylswyk et al., 1997). If the colonization of this phylum is inhibited, it's questionable how efficient these animals will

be in production if energy sources are few. *Bifidobacterium* is also involved in initial establishment in the GIT with probiotic actions exerted on the host, especially infants as this phylum is attuned to metabolism of milk oligosaccharides (O'Callaghan et al., 2016). *Christensenella* has been shown to be the most heritable of the gut bacteria and is directly related to obesity. This phylum has been found in higher abundance in individuals with a lower body mass index and combats obesity by decreasing adipose deposits (Madsen et al., 2017). The *Sphingomonas* phylum dwindled in the AF diet as well and has been shown to contribute to the immune response by producing glycolipids for natural killer T cells that ward off infection (Caballero et al., 2015). From a beneficial standpoint, it was observed that there was a downregulation of *Chlamydia* that resides in the gut as an opportunistic pathogen (Rank et al., 2014). Once infection ensues, the immune system is unable to clear the *Chlamydiae* entirely, thus a lower initial presence is desirable. The same concepts apply for *Actinobacillus*, as this phylum has been related to numerous fatal diseases and is inactively harbored in the GIT until infection is triggered (Rycroft et al., 2000). The AFNS diet showed increases in abundance of bacteria more than any other diet. Among these phyla was *Ruminococcaceae* that has been associated with individuals that maintain high energy metabolism (Menni et al., 2017). *Allisonella* has been shown to increase insulin resistance and tumor necrosis factor, leading to endotoxemia and small intestinal bacterial growth (Catinean et al., 2018). *Collinsella* was upregulated in the AFNS diet and can alter the host bile acids to regulate the virulence and pathogenicity of enteric pathogens (Gomez-Arango et al., 2018). *Leptotrichia* and *Enterococcus* are other opportunistic pathogens that mainly reside in the oral cavity and ferment carbohydrates to produce lactic acid in both the mouth and the GIT (Eribe et al., 2008). Species of the

genus *Slackia* that were found in high abundance are known to produce equol from isoflavones in the human intestine (Jin et al., 2009). If more equol producing bacteria are present where supplemental isoflavones have greater inclusion, there is more opportunity for utilization of the metabolites and ability to study the effects. Upregulation of *Syntrophococcus* was observed, a phylum able to metabolize isoflavones and was present in the diet containing supplemented isoflavones. These bacteria have been shown to participate in *O*-demethylation of monoaromatic derivatives in the rumen, such as the formation of *O*-DMA (Doré et al., 1990). Greater abundance of isoflavone metabolizing microbes was shown in the diet that included supplemental isoflavones, proposing that these bacteria will colonize in the microbiome if isoflavones are included in diet in the early stages of life.

Alpha diversity as shown in Figures 2.1 and 2.3 identifies differences in the number of species present in day and sample location; however, there were evidently similar numbers of bacteria in diet, as shown in Figure 2.2. *Euryarchaeota* are of interest as they are organisms known to survive in even the most extreme conditions, potentially able to thrive when disease occurs (Castro-Fernandez et al., 2017). *Planctomycetes*, *Lentisphaerae*, and *Verrucomicrobia* that metabolize methane were shown to increase over time, highlighting the diversity that increases in the microbiome in even just two weeks (Lee et al., 2009). The integrity of the microbiome was enhanced as *Actinobacteria* increased, a prominent member of the gut microbiome that produces antibiotics (Ventura et al., 2007), providing a defense mechanism for both the bacteria and the host. The microbiome diversity continued to proliferate with more of the prominent genus', such as *Cyanobacteria* and *Fibrobacteres* becoming more abundant

with the change from the phase 1 to phase 2 diets. *Fibrobacteres* participates in cellulose metabolism and as more fiber was included in the diet, the more opportunity for this genus to thrive (Ransom-Jones et al., 2012). The increase of *Chlamydiae* showed that beneficial bacteria weren't the only ones able to colonize, as this opportunistic pathogen was able to gain access to the GIT and find its niche (Rank et al., 2014).

Epsilonbacteraeota have been shown to be early colonizers in previous studies (Methou et al., 2019) as symbiotic bacteria, observations verified with the indication of high abundance in the nursery phase of piglets. There was an upsurge of

Epsilonproteobacteria, *Patescibacteria*, and *Deferribacteres*, genus' known for the utilization of iron and reduction of nitrate (Gittel et al., 2012), showing how the microbes are able to break down more material in the gut as more bacteria colonize. *Tenericutes* further shows the increase in diversity by being one of the few bacteria that do not possess a peptidoglycan cell wall (Skenner et al., 2016). An increase in the abundance and diversity of these vastly different bacteria represent how much the microbiome can develop in a short time period.

Sample location was shown to affect microbial composition, especially in the colon where luminal contents were much less diverse than colonic mucosa. In the digesta, there were many genus's accounted for that were in less abundance, including both beneficial and pathogenic bacteria. It is understandable that more bacteria are present in the lining, the greater opportunity for colonization compared to the digesta that is constantly moving through the GIT until it is expelled from the body, thus bacteria must constantly be competing for a spot in the lining to maintain their niche and stay in the body. Some of the bacteria in the digesta could be present only due to the sloughing of

the intestinal walls that force them into the lumen. It is worth noting that the fecal samples were similar to the colonic digesta samples, thus feces may be used for microbial analysis of the colonic digesta that uses a less invasive method for collection.

IL-8 circulating in the blood serum was not different among diets; however, it did increase over time. Studies have shown that as mammals mature, their immune systems are more suited to focus on stimulating factors that give the organism an advantage against potential pathogens by promoting synthesis and secretion of cytokines (Carstensen et al., 2010). While IL-8 may not have been mobilized with the inclusion of isoflavones, other cytokines and TNF- α concentrations would be of interest, because only 1 cytokine does not mean an increase or decrease in gut health or immunity.

CONCLUSION

There were no differences in growth performance or circulating IL-8 among diet ($P > 0.10$) for this experiment; however, there is evidence that diet, time, and location of sample extraction all affect the gut microbiome composition. Arcon-F, the diet with essentially no inclusion of isoflavones decreased in microbial diversity compared to the other diets ($P < 0.001$), while the Arcon-F + Novasoy 400 diet had greater concentrations of notable bacterium, including those that can metabolize isoflavones into equol ($P < 0.01$). The HP-300 and HP-300 + Novasoy 400 diets did not show any significant differences within this threshold of analysis. Between d 0 and 14 of the experiment, there was a shift in the microbiota with significant changes even in the first phase as a result of the switch in diet ($P < 0.001$). This suggests that the microbiome is easily affected during the first stages of life and is rapidly changing during this time with the introduction of

new feed and/or a new environment. The location in the gastrointestinal tract has varying microbial composition as well, as mucosa and digesta samples differ considerably ($P < 0.05$). However, feces and colonic digesta are close enough in composition that if one is interested in CD microbial structure, taking fecal samples will suffice as a less invasive method. A decrease in beneficial bacteria in the GIT of pigs fed soy protein concentrate diet with no isoflavones suggests that some species of bacteria thrive off of the phytoestrogens and other compounds typically found in SBM that are removed during processing. These bacteria may be involved in gut barrier function and integrity. Further experimentation should take place with a disease challenge where piglets are infected with a pathogen after ingesting varying doses of isoflavones and immune parameters and the microbiome again analyzed.

IMPLICATIONS

During wk 4, there was a decrease in ADFI and ADG compared to wk 3. This is not typical to experimentation as growth and feed intake usually only increase with the duration of the experiment. There could also be upregulation or downregulation of bacteria in the diets that were not included in the top 10 families; however, our analysis only included those families most prominent.

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TABLES

Table 2.1. Ingredient composition (% as-fed bases) and calculated nutrient composition of diets for Phase 1.

	Diets			
	HP-300	HP-300 + Novasoy 400	Arcon-F	Arcon-F + Novasoy 400
Ingredients, %				
Corn	42.3	41.9	42.5	41.9
Dicalcium phosphate, 18.5% P	1.05	1.05	1.10	1.10
Limestone	1.07	1.07	1.00	1.00
Salt	0.10	0.10	0.10	0.10
Vitamin premix ¹	0.25	0.25	0.25	0.25
Trace mineral premix ²	0.15	0.15	0.15	0.15
Corn Oil	3.00	3.00	3.00	3.00
Lysine·HCl	0.45	0.45	0.21	0.21
DL-Methionine	0.14	0.14	0.32	0.32
L-Threonine	0.11	0.11	0.02	0.02
Whey, dried	15.2	15.2	15.2	15.2
Dairylac-80	11.3	11.3	11.3	11.3
HP-300	25.0	25.0	0.00	0.00
Novasoy 400	0.00	0.35	0.00	0.50
Arcon-F	0.00	0.00	25.0	25.0
Calculated nutrient composition, %				
Crude protein	29.7	29.7	22.5	22.5
ME (kcal/kg) ³	3530	3518	3037	3037
Ca	0.80	0.80	0.80	0.80
STTD ⁴ P	0.40	0.40	0.40	0.40
SID ⁴ Lys	1.35	1.35	1.36	1.36
SID Met	0.40	0.40	0.40	0.40
SIDl Thr	0.79	0.80	0.80	0.80
SID Trp	0.23	0.23	0.23	0.23
Total Lysine	1.48	1.48	1.40	1.40
Total Phosphorus	0.62	0.61	0.62	0.62
Ca:P	1.29	1.31	1.29	1.29
Isoflavones (mg/kg)	604	2011	0	2011

¹Vitamin premix supplied per kg of diet: vitamin A (as retinyl acetate), 5500 IU; vitamin D (as cholecalciferol), 550 IU; vitamin E (as tocopherol acetate), 30 IU; vitamin K (as menadione dimethylpyrimidinol bisulfate), 4.4 mg; riboflavin, 11.0 mg; d-pantothenic acid, 22.05 mg; niacin, 33.0 mg; vitamin B₁₂ (as cyanobalamin), 33.0 mg.

²Trace mineral premix: copper (as CuSO₄·5H₂O), 10 mg/kg; iodine (as Ca(IO₃)·H₂O), 0.25 mg/kg; iron (as FeSO₄·2H₂O), 125 mg/kg; manganese (MnO), 15 mg/kg; selenium (Na₂SeO₃), 0.3 mg/kg; zinc (ZnSO₄·H₂O), 125 mg/kg.

³ME: metabolizable energy

⁴STTD: standardized total tract digestible; SID: standard ileal digestibility

Table 2.2. Ingredient composition (% as-fed bases) and calculated nutrient composition of diets for Phase 2.

	Diets			
	HP-300	HP-300 + Novasoy 400	Arcon-F	Arcon-F + Novasoy 400
Ingredients, %				
Corn	45.7	45.3	45.9	45.4
Dicalcium phosphate, 18.5% P	0.70	0.70	0.70	0.70
Limestone	1.07	1.07	1.00	1.00
Salt	0.10	0.10	0.10	0.10
Vitamin premix ¹	0.25	0.25	0.25	0.25
Trace mineral premix ²	0.15	0.15	0.15	0.15
Corn Oil	3.00	3.00	3.00	3.00
Lysine-HCl	0.41	0.41	0.20	0.20
DL-Methionine	0.13	0.13	0.28	0.28
L-Threonine	0.11	0.11	0.02	0.02
Whey, dried	15.2	15.2	15.2	15.2
Dairylac-80	11.3	11.3	11.3	11.3
HP-300	22.0	22.0	0.00	0.00
Novasoy 400	0.00	0.37	0.00	0.50
Arcon-F	0.00	0.00	22.0	22.0
Calculated nutrient composition, %				
Crude protein	27.1	27.1	20.8	20.8
ME ³ , kcal/kg	3536	3524	3103	3103
Ca	0.70	0.70	0.70	0.70
STTD ⁴ P	0.33	0.33	0.33	0.33
SID ⁴ Lys	1.24	1.23	1.24	1.24
SID Met	0.37	0.37	0.36	0.36
SID Thr	0.74	0.74	0.74	0.74
SID Trp	0.20	0.20	0.21	0.21
Total Lysine	1.37	1.35	1.36	1.36
Total Phosphorus	0.55	0.53	0.53	0.53
Ca:P	1.27	1.32	1.32	1.32
Isoflavones (mg/kg)	532	2019	0	2019

¹Vitamin premix supplied per kg of diet: vitamin A (as retinyl acetate), 5500 IU; vitamin D (as cholecalciferol), 550 IU; vitamin E (as tocopherol acetate), 30 IU; vitamin K (as menadione dimethylpyrimidinol bisulfate), 4.4 mg; riboflavin, 11.0 mg; d-pantothenic acid, 22.05 mg; niacin, 33.0 mg; vitamin B₁₂ (as cyanobalamin), 33.0 mg.

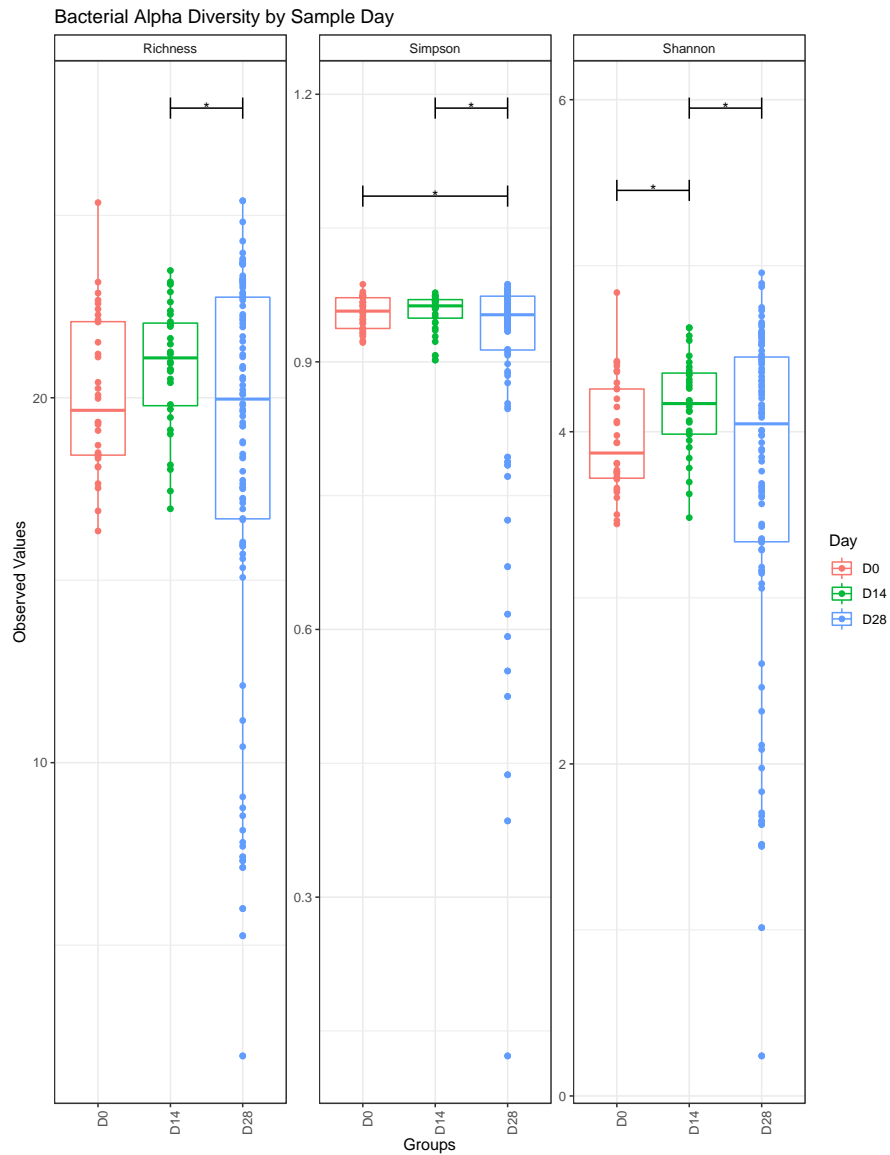
²Trace mineral premix: copper (as CuSO₄·5H₂O), 10 mg/kg; iodine (as Ca(IO₃) · H₂O), 0.25 mg/kg; iron (as FeSO₄ · 2H₂O), 125 mg/kg; manganese (MnO), 15 mg/kg; selenium (Na₂SeO₃), 0.3 mg/kg; zinc (ZnSO₄ · H₂O), 125 mg/kg.

³ME: metabolizable energy

⁴STTD: standardized total tract digestible; SID: standard ileal digestibility

FIGURES

Figure 2.1. Bacterial alpha diversity over time for days 0, 14, and 28.



Richness: the number of species per sample; The more species present in a sample, the 'richer' the sample.

Simpson alpha diversity index: the number of organisms of a particular species (n) over the total number of organisms of all species (N) subtracted from 1.

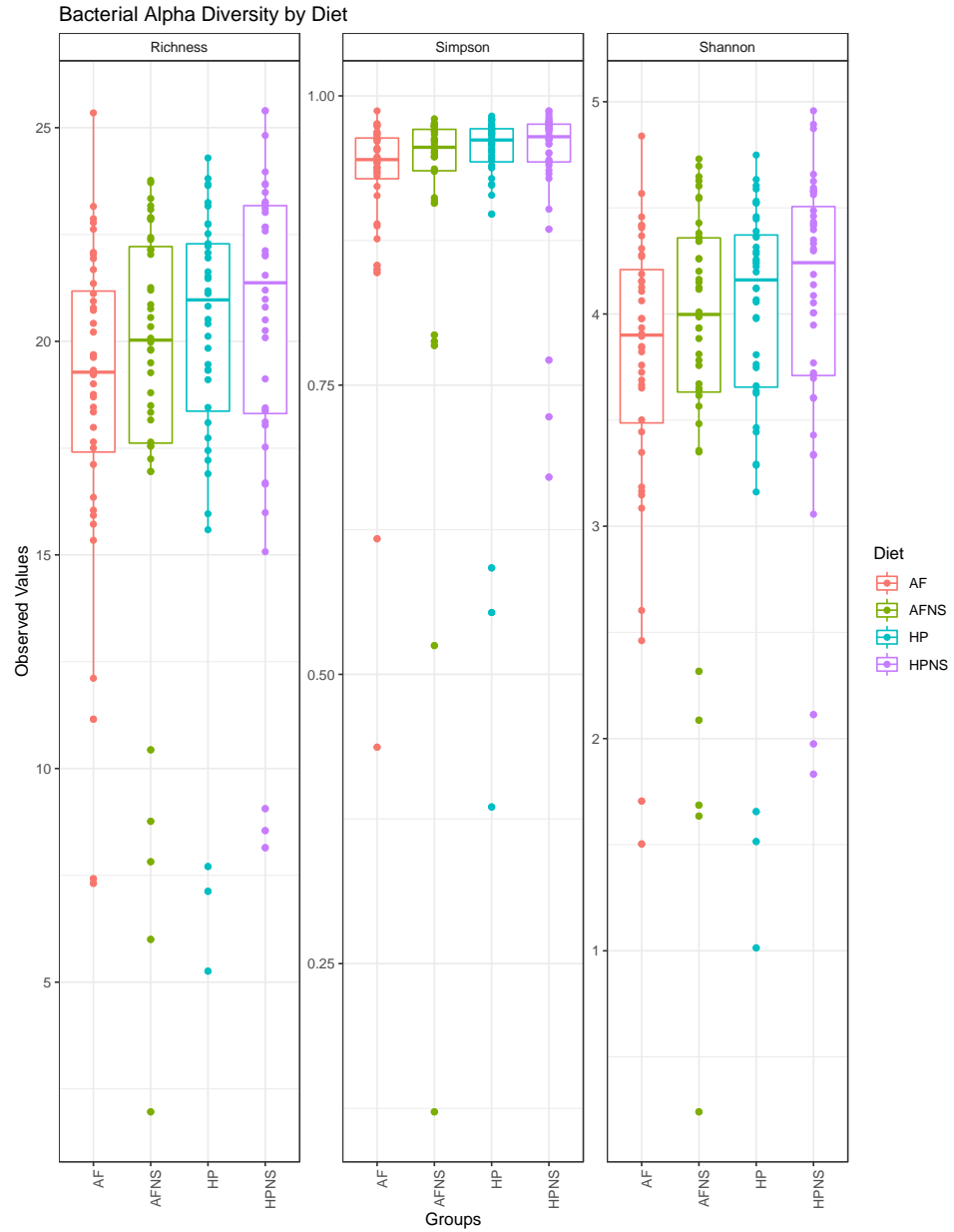
$$D = 1 - \left(\frac{\sum n(n-1)}{N(N-1)} \right)$$

Shannon diversity index: the calculated proportion of species i relative to the total number of species (p_i), multiplied by the natural logarithm of this proportion ($\ln p_i$), multiplied by -1.

$$H = - \sum_{i=1}^S p_i \ln p_i$$

(*) = ($P < 0.05$)

Figure 2.2. Bacterial alpha diversity for overall experimental diets.



Richness: the number of species per sample; The more species present in a sample, the 'richer' the sample.

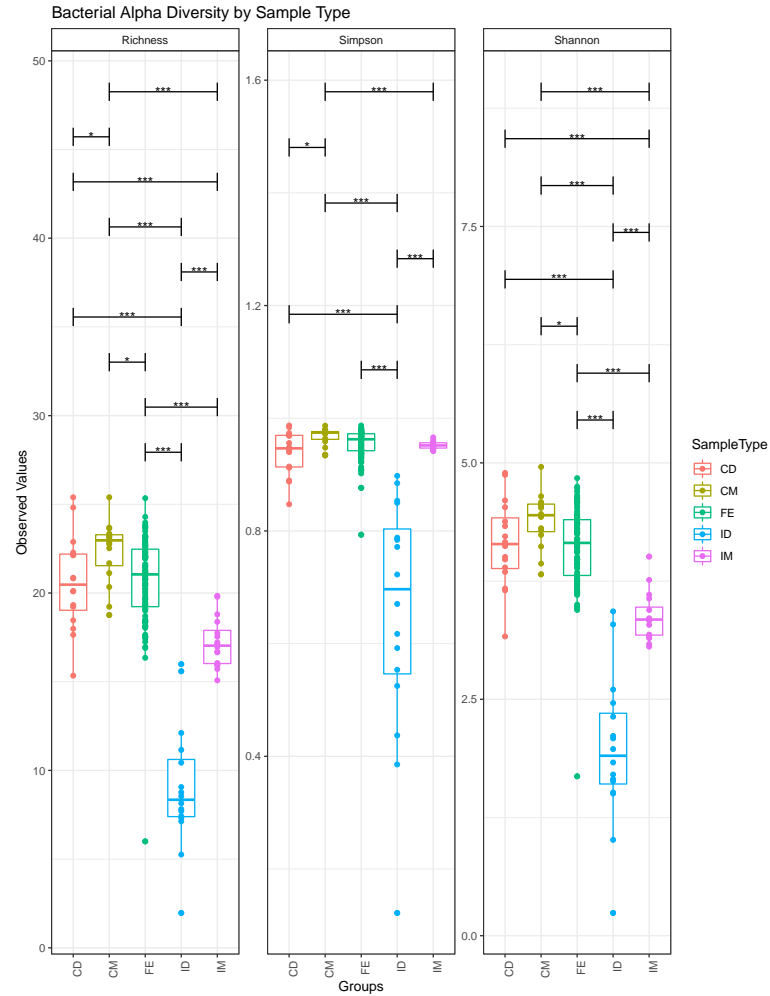
Simpson alpha diversity index: the number of organisms of a particular species (n) over the total number of organisms of all species (N) subtracted from 1.

$$D = 1 - \left(\frac{\sum n(n-1)}{N(N-1)} \right)$$

Shannon diversity index: the calculated proportion of species i relative to the total number of species (p_i), multiplied by the natural logarithm of this proportion ($\ln p_i$), multiplied by -1.

$$H = - \sum_{i=1}^S p_i \ln p_i$$

Figure 2.3. Bacterial alpha diversity compared between sample locations (SampleType).



Richness: the number of species per sample; The more species present in a sample, the 'richer' the sample.

Simpson alpha diversity index: the number of organisms of a particular species (n) over the total number of organisms of all species (N) subtracted from 1.

$$D = 1 - \left(\frac{\sum n(n-1)}{N(N-1)} \right)$$

Shannon diversity index: the calculated proportion of species i relative to the total number of species (p_i), multiplied by the natural logarithm of this proportion ($\ln p_i$), multiplied by -1.

$$H = - \sum_{i=1}^S p_i \ln p_i$$

(*) = P < 0.05, (***) = P < 0.001

Figure 2.4. Principal Component of Analysis plot for time of sample collection versus diet.

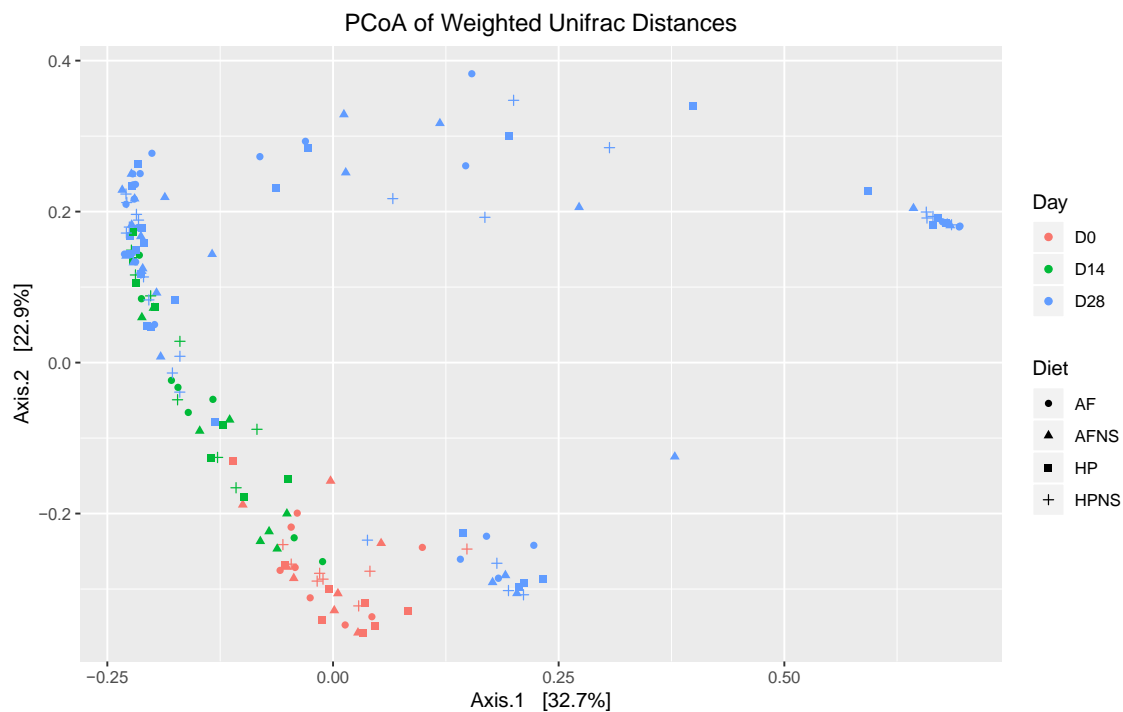


Figure 2.5. Principal Component of Analysis plot for time of sample collection versus location of sample.

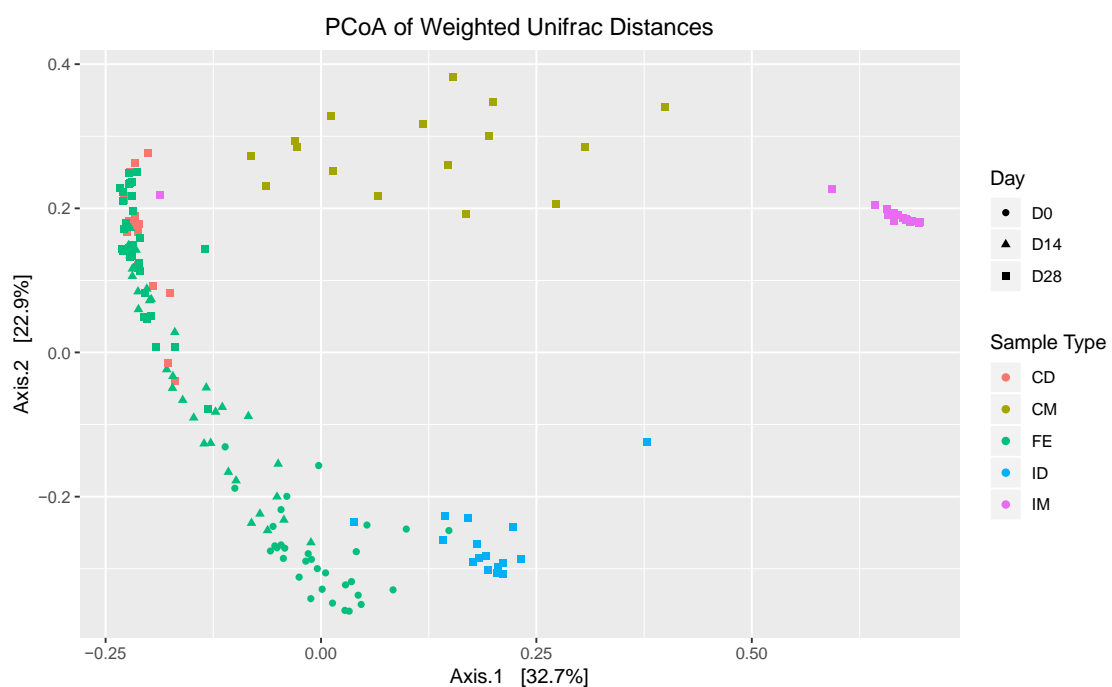


Figure 2.6. Principal Component of Analysis plot for diet versus location of sample.

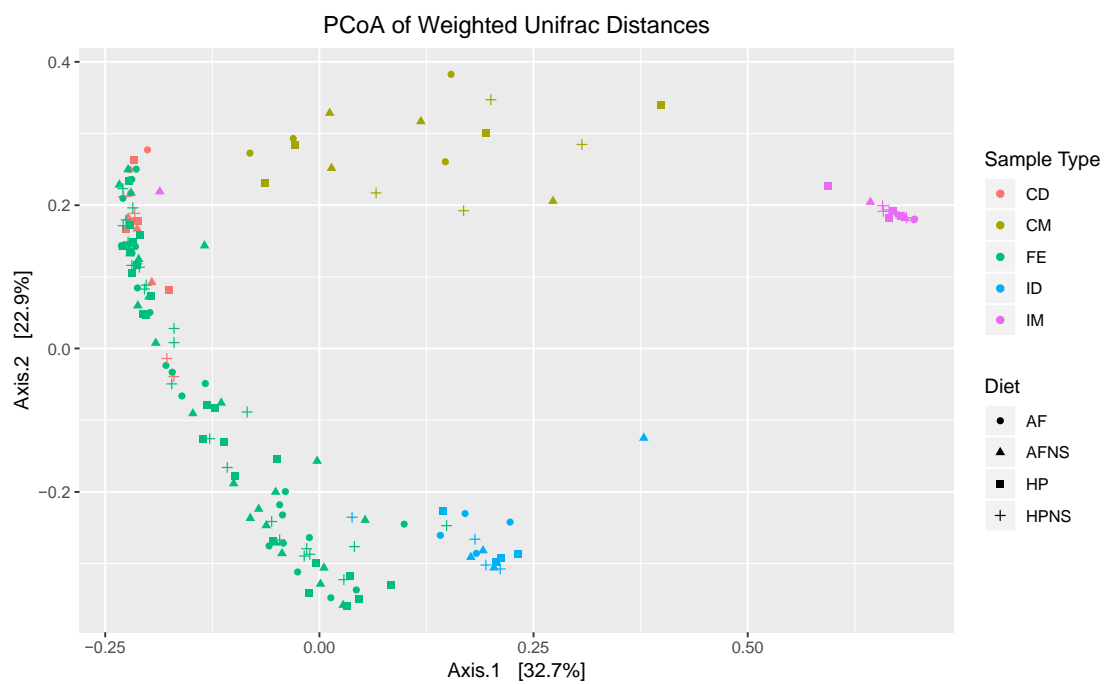


Figure 2.7: ELISA results of diet over time in pg/mL.

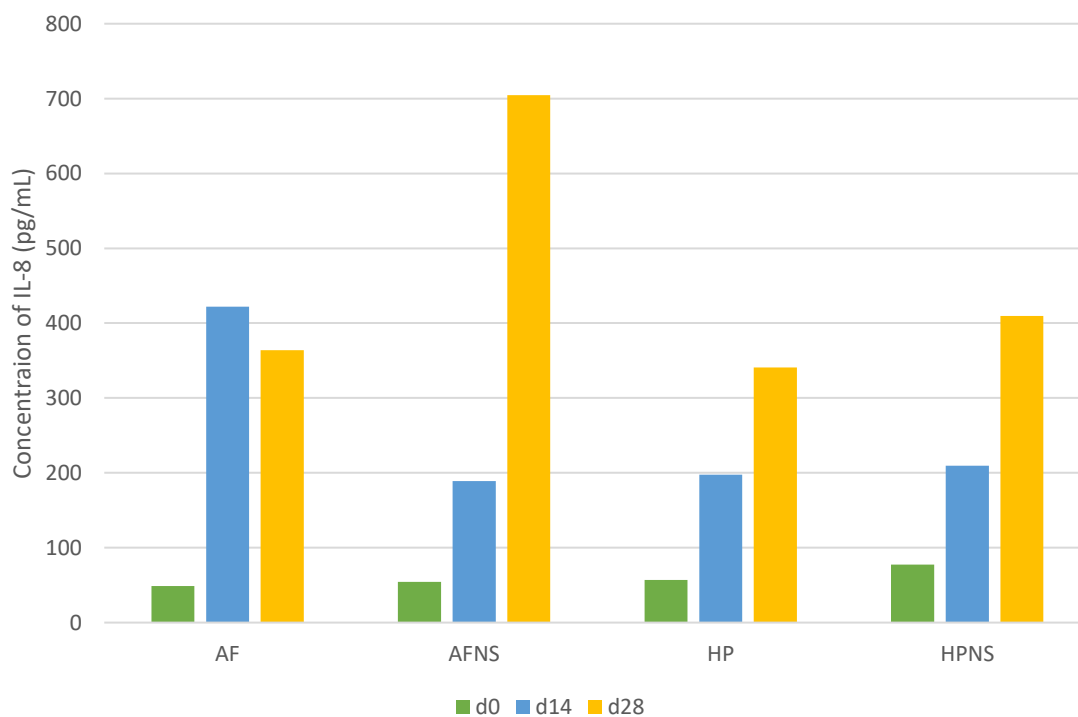


Figure 2.8. Phylum composition of sampling site bacterial communities as shown within diet.

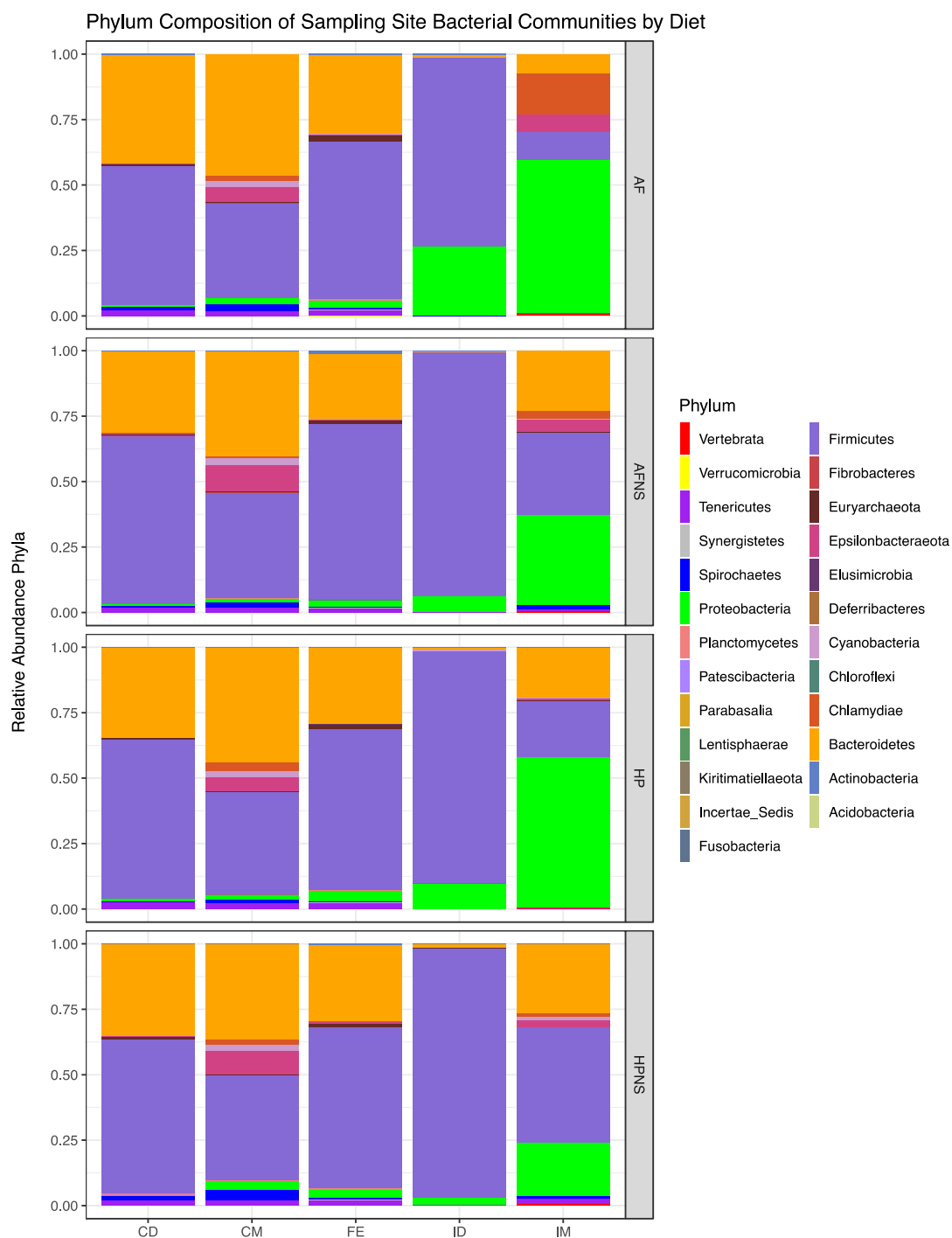


Figure 2.9. Top 10 species diversity in diets.

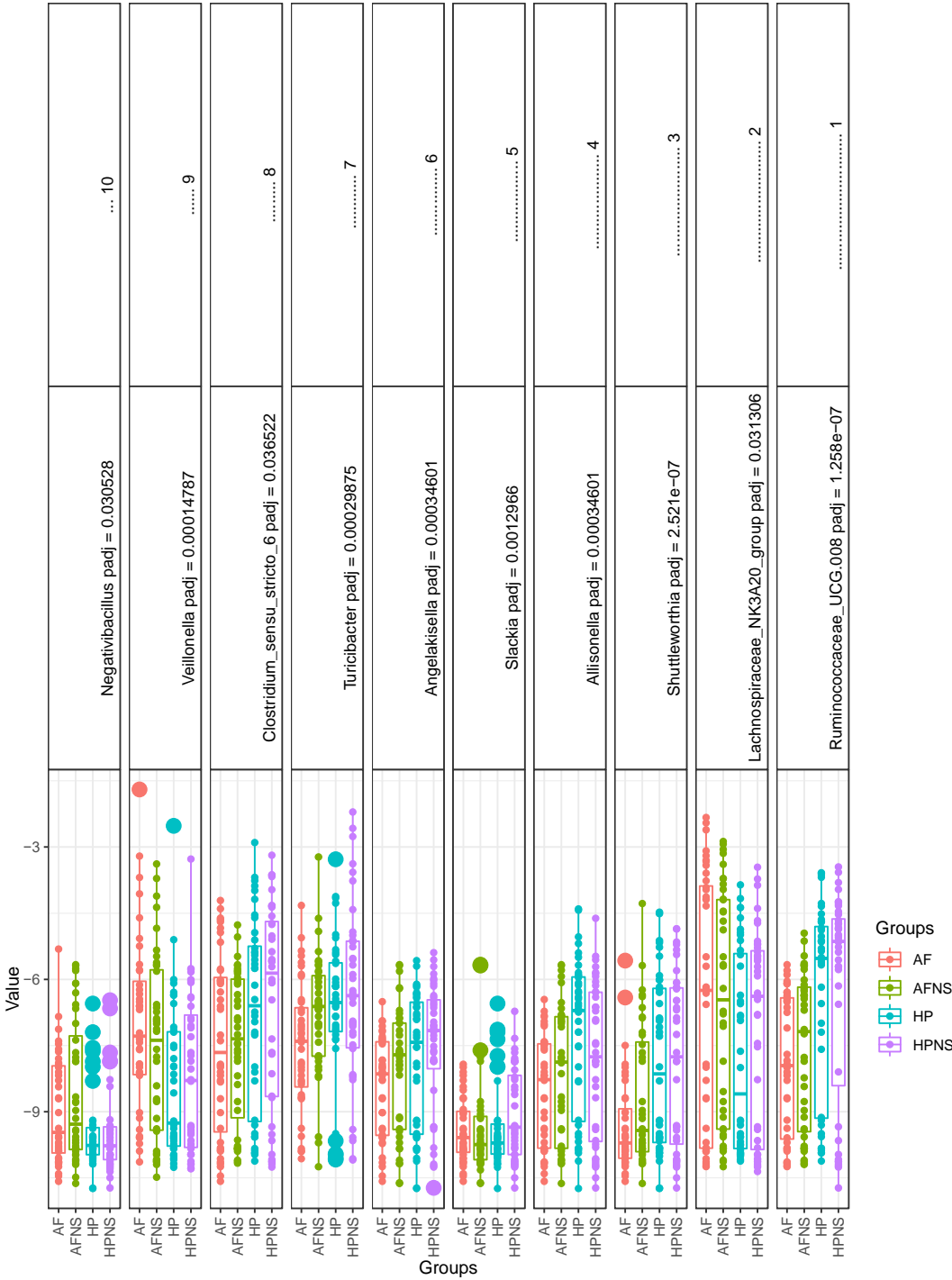


Figure 2.10. Average daily gain within diet in wk 1-4, phase 1-2, and overall.

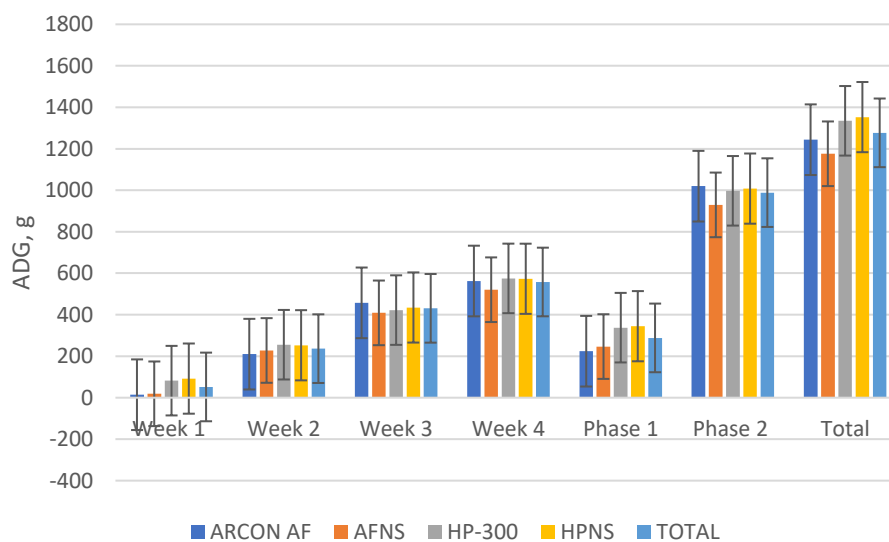


Figure 2.11. Average daily feed intake within diet in wk 1-4, phase 1-2, and overall.

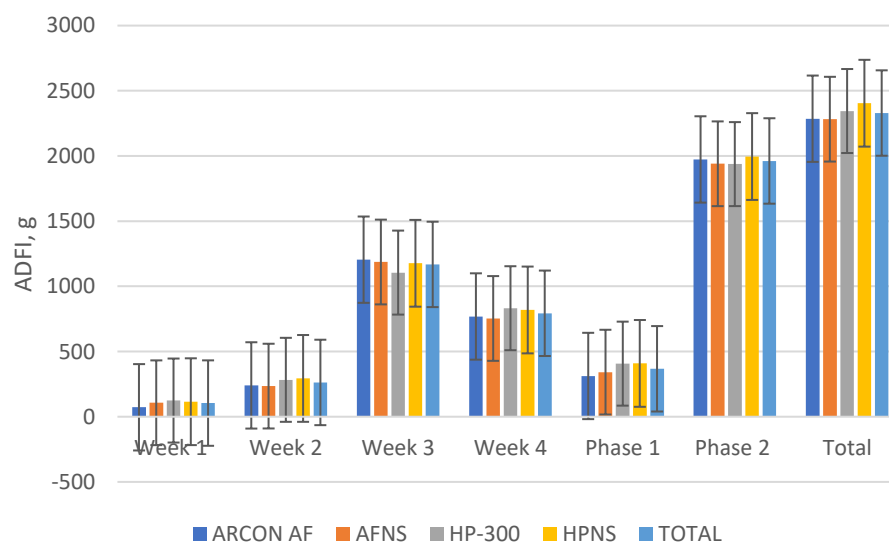


Figure 2.12. Gain:feed within diet in wk 1-4, phase 1-2, and overall.

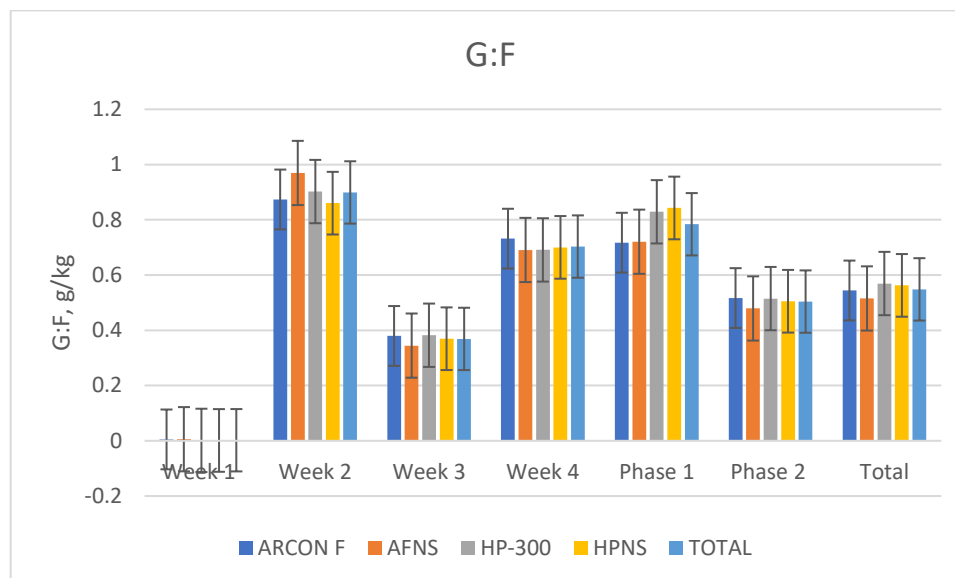
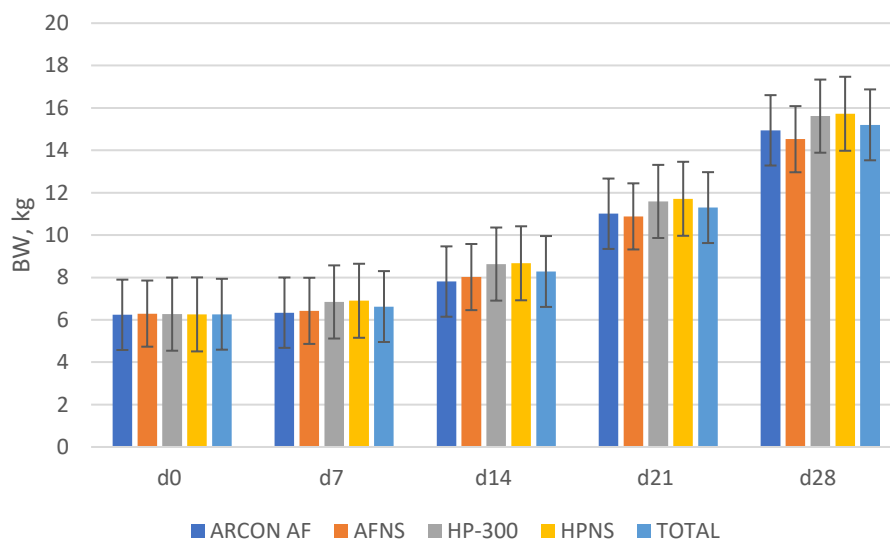


Figure 2.13. Body weight within diet on d 0, 7, 14, 21, and 28.



Chapter 4: Determination of immune responses in an intestinal epithelial cell line treated with varying doses of genistein and the individual isoflavones.

ABSTRACT

Two experiments were conducted where IPEC-J2 cells were treated with varying concentrations of the isoflavone genistein or similar concentration of each of the isoflavones genistin, genistein, daidzin, and daidzein. In the first experiment, cells were split into 12 6-well plates with 50,000 cells per well in transmembrane wells and treated with varying concentrations of genistein (0, 50, 150, and 300 μM), lipopolysaccharides (LPS), or polyinosinic:polycytidylic acid (poly I:C). In the second experiment, cells were split into 12 6-well plates with 75,000 cells per well and treated with individual isoflavones at a concentration of 50 μM , while poly I:C was included as well as diclofenac sodium salt. In both experiments, media was extracted from the basolateral membrane (BLM) and apical membrane (AM) for IL-8 ELISA analysis after plates were incubated at 37° C and 5% CO₂ at hours 0, 3, 6, and 12. Transepithelial electrical resistance (TEER) was measured on the plates that were used for hour 12 collection of media. In Exp. 1, treatment did not cause a difference in IL-8 ($P > 0.10$), but there was an increase in IL-8 present in the media with time ($P < 0.001$) and between BLM and AM ($P < 0.005$). Treatment did cause differences in Exp. 2 ($P = 0.0186$) where poly I:C secreted more IL-8 overall and daidzin was the most anti-inflammatory isoflavone in this experiment, suggesting that it is rapidly converted into daidzein in the intestinal membrane.

In both Exp. 1 and 2, there were no differences for overall TEER ($P > 0.10$). In Exp 1., LPS produced a greater epithelial resistance by hour 12 ($P = 0.014$) than the other treatments. The genistein treatments did not produce greater TEER than LPS; however, all 3 genistein treatments averaged greater TEER than poly I:C and 0 μM . The 50 μM

genistein treatment had greater TEER and IL-8 secretion than 150 or 300 μM , thus this concentration was used in Exp. 2 to for optimize membrane integrity. In both experiments, more IL-8 was secreted into the BLM, which simulates the barrier between the enterocytes and the blood ($P < 0.01$). In both experiments, there was an increase in IL-8 secretion over time ($P < 0.05$), imitating production of the chemokine in the GIT. It was determined that 50 μM of isoflavones is an adequate concentration to treat cells with for immune studies. In Exp. 2, the isoflavones did not produce as great of concentration of IL-8 as the poly I:C, suggesting that they prevent inflammatory responses from occurring. They also did not degrade the epithelial lining to the point of dysfunctionality and thus may be used in IPEC-J2 cell line experiments.

Key words: genistein, interleukin-8, IPEC-J2, transepithelial electrical resistance

INTRODUCTION

Soy as a protein source is becoming increasingly popular in Western diets due to the discovery of numerous benefits associated with the high soy content in Asian diets. Soybean meal is a popular protein source in swine diets due to low cost and high digestibility. In soy, estrogen-shaped compounds known as isoflavones are found as daidzin, genistin, and glycitin, the glycosidic forms (Izumi et al., 2000). Isoflavones have been shown to influence immune parameters in mammals; however, the appropriate composition of the gut microbiome is required to produce the desired metabolites that elicit such responses. Found in soy, isoflavones are still commonly considered to be anti-nutritional factors because they have been shown to increase cell proliferation in some cases, causing quicker progression of some cancers (Khan et al., 2012). Daidzein and genistein are the active forms that are cleaved in the gastrointestinal tract from their glycosidic forms, daidzin and genistin, by β -glycosidases (Yu et al., 2016). Isoflavones have been proven to impact immunity and influence the concentration of circulating cytokines in the blood while influencing gut barrier function and membrane integrity (Gilbert et al., 2013). Genistein has been proven to retain antiviral, anti-inflammatory, and antimicrobial properties and is known as the bioactive isoflavone (Chinta et al., 2012). Structurally similar to estrogen, genistein exerts estrogenic and anti-estrogenic activity on the host. While many characteristics of genistein are desirable, studies have shown either drastic upregulation or downregulation of cell proliferation in incidences that either damage crypt cells in the jejunum or increase proliferation to the point that cancer occurs (Dong et al., 2010). Daidzein forms the metabolite S-equol that is more estrogenic and a more potent antioxidant than daidzein that binds to estrogen receptors

with more affinity (Kobayashi et al., 2013). To better understand how to take advantage of the anti-inflammatory, antiviral, and antimicrobial properties that have been found in some cases, research must take place to determine the mechanisms of immune responses elicited by the isoflavones individually and as a mixture. It has been reported that isoflavones are involved in interactions that enrich the immune response (Yu et al., 2016); however, individual isoflavone responses should be characterized first to understand the contribution that each makes to the innate immune system. IPEC-J2 cells are a non-transformed epithelial cell line cultured from the jejunum of an unsuckled neonatal piglet. These cells are ideal to measure membrane integrity and cytokine production before introducing to a live animal (Brosnahan et al., 2012). These cells mimic the intestinal wall lining when cultured in transmembrane well inserts, including a basolateral membrane side (BLM) that represents the barrier between enterocytes and the blood, while the apical membrane (AM) symbolizes the membrane between the cells and the lumen (Chopra et al., 2010). The innate immune system will secrete pro-inflammatory and anti-inflammatory cytokines into both the BLM and AM, and IPEC-J2 cells have been found to secrete both into the media on either side of the transmembrane well insert. They are capable of being models for the intestinal lining as they are robust cells that can maintain function up to 100 passages and still produce immune responses when treated with compounds of interest, such as genistein (Vergauwen et al., 2015). There are typical concentrations of genistein in products such as soy-based infant formulas and soybean meal; however, supplementation of excess isoflavones, including genistein, is of interest and determination of the threshold appropriate to include is still being researched. In order to harness the benefits without experiencing the adverse

effects of isoflavones, different doses of isoflavones both individually and mixed must be explored. The IPEC-J2 cell line is useful for determination of maximum and minimum treatment exposure while using a more cost-effective method than an in vivo trial, first.

Objective: To determine the appropriate concentration of genistein to use as a baseline in cell culture experiments with IPEC-J2 cells and other isoflavones while maintaining intestinal integrity through measurements of cytokine production and transepithelial electrical resistance to rationalize use of treatments in further research, including disease challenges and observing the immune effects of other isoflavones.

MATERIALS AND METHODS

Cell culture

IPEC-J2 cells were brought to confluency in two 75 cm² flasks and split into n = 12 6-well plates with 0.4 µm pore transmembrane well cell culture inserts (Falcon, Corning, NY) in a density of 50,000 cells per well in Exp. 1 and 75,000 cells in Exp. 2. Cells were brought to ≥95% confluency before media was aspirated, washed with PBS, and treated for 1 hour with the designated treatments. Once the hour was over, media was again aspirated and cells were washed with PBS before being cultured with a 1:1 ratio of Dulbecco's Modified Eagle Medium (ThermoFischer Scientific, Waltham, MA) and Ham's F-12 Nutrient Mixture (Gibco, ThermoFischer Scientific, Waltham, MA), 10% fetal bovine serum (FBS), 10 µL/ 2.0 L epidermal growth factor (EGF), 1% penicillin/streptomycin, and 1% insulin/transferrin/Na selenite media supplement.

Treatments

In Exp. 1, cells were treated with 4 concentrations of genistein (from *Glycine max*, soybean, ~98% HPLC, Sigma-Aldrich, St. Louis, MO) at 0, 50, 150, or 300 μ M. These treatments were determined by the average concentration of isoflavones in a typical soybean meal diet commonly used in the industry and supplementation up to 6 times the normal dose. These concentrations have commonly been found to lie within a margin of safety where the deleterious effects of isoflavones do not normally occur. The remaining cells were exposed to either 10 ng/ μ L lipopolysaccharides (LPS) or 20 μ g/mL polyinosinic:polycytidylic acid (poly I:C). The 0 μ M treatment was used as a control while LPS and poly I:C were used as negative controls since they are known to elicit innate immune system responses and possess pro-inflammatory properties. In Exp. 2, cells were treated with 50 μ M of either genistein (from *Glycine max*, soybean, ~98% HPLC), genistin (from *Glycine max*, soybean, \geq 95% HPLC), daidzein (\geq 98%, synthetic), and daidzin (\geq 95% HPLC) (Sigma-Aldrich, St. Louis, MO). Cells were also treated with 0.2 mM diclofenac sodium salt as a positive control. 20 μ g/mL polyinosinic:polycytidylic acid (poly I:C) was used as a negative control in the final well of the 6-well plate. Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) and is appropriate to use as a positive control while poly I:C was used as a negative control as it produces pro-inflammatory cytokines in a natural setting.

Sample collection and analysis

Samples were collected at hours 0, 3, 6, and 12 from 3 plates in the BLM and AM while TEER was measured in the 3 plates that were used through hour 12. TEER was

measured using an EVOM² Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL) with STX2 chopstick electrodes. The electrodes were placed on each side of the well insert membrane and measured in ohms. Samples were stored in 1.5 mL microcentrifuge tubes at -20 °C until used for ELISA. A Human IL-8/CXCL8 DuoSet ELISA and DuoSet ELISA Ancillary Reagent Kit 2 were used on samples that were plated in duplicate to enhance reproducibility and ensure accuracy of the spectrometer. A FLUOstar Omega microplate spectrometer was used to determine concentration of IL-8 in the wells in picograms/microliter. Both IL-8 and TEER values were analyzed for differences using a repeated measures analysis of variance (ANOVA) for TEER and one-way ANOVA for IL-8 in R (R studio, 3.6.0, The R Project) to differentiate treatment effects at a significance level of $P \leq 0.05$ and trends between $0.05 < P > 0.15$.

IL-8 ELISA

A DuoSet ELISA Porcine IL-8/CXCL8 kit was used to measure porcine Interleukin 8 in serum isolated from blood. All reagents were brought to room temperature before use and components were allowed to sit for a minimum of 15 minutes with gentle agitation after initial reconstitution. Working dilutions were prepared and used immediately, except for the Wash Buffer and Reagent Diluent. The Capture Antibody was diluted to working concentration in PBS without the carrier protein and immediately used to coat a 96-well microplate, sealed, and incubated overnight at room temperature. The next day, wells were aspirated and washed with Wash Buffer 3 times using a manifold dispenser. The plate was then blocked with Reagent Diluent and incubated at room temperature for at least 1 hour and washed again. The samples were

then added to the wells in duplicate to enhance reproducibility, covered with an adhesive strip, and incubated at room temperature for 2 hours. The plate was then washed before the Detection Antibody, diluted in Reagent Diluent and Normal Goat Serum, was added to each well, covered with a new adhesive strip, and incubated for 2 hours at room temperature. Samples were then washed before a working dilution of Streptavidin-HRP was added to each well and incubated for 20 minutes at room temperature while covered and out of direct light. The wash step occurred once more before the Substrate Solution was added to each well incubated for 20 minutes at room temperature out of direct light. The Stop Solution was then added to each well and the plate was gently tapped to ensure thorough mixing before the optical density of each well was determined. The microplate reader was set to 450 nm and wavelength correction at 540 nm.

RESULTS

Interleukin-8

In Exp 1., IL-8 did not differ between treatments ($P > 0.10$); however, secretion of IL-8 overall increased over time ($P < 0.001$) as shown in Figure 4.1. There was also a greater concentration of IL-8 secretion in the BLM when compared to the AM ($P < 0.001$).

In Exp. 2, the poly I:C treatment produced the greatest secretion of IL-8 of the treatments ($P = 0.0186$) while daidzin stimulated the least amount of release, as shown in Figure 5.1. The more that time went on, the more IL-8 that was released into the media with the greatest increase in cytokine production taking place between hours 6 and 12 (P

< 0.001). There was an increase in the concentration of IL-8 as samples were taken from the basolateral membrane ($P = 0.016$).

Transepithelial electrical resistance

In Exp. 1, TEER proved to vary both among treatments and over time. LPS produced greater membrane integrity ($P = 0.0137$) than the other treatments, while 150 μM of genistein produced the lowest. Intestinal integrity increased considerably over time ($P = 0.0107$) with the 6-hour time interval between hours 6 and 12 showing a dramatic increase in ohms.

In Exp. 2, there was not a difference in TEER among treatments ($P > 0.10$), nor were there any differences over time ($P > 0.10$). This is illustrated in Figure 5.2 where the electrical resistance is measured and represented in ohms against time samples were collected on hours 0, 3, 6, and 12.

DISCUSSION

Genistein did not prove to be more proactive in IL-8 secretion than the LPS; however, it was also not lower than the 0 μM control or poly I:C negative control. There were not statistically significant differences between treatments; however, Figure 4.2 shows that LPS increased substantially faster than the other treatments if more time was allowed with the 50 μM genistein treatment closely behind. 50 μM genistein produced better IL-8 secretion in the BLM and AM compared to 150 μM and 300 μM , suggesting that a lower concentration more closely related to genistein concentrations in industry products should suffice for further in vitro analysis without degrading the cells. BLM had

more IL-8 secretion than AM as expected, reaffirming that this cell line is useful in determining how the concentrations of cytokines released into the blood circulation operate instead of being wasted in the lumen. The LPS treatment was greater in TEER while 50 μM genistein was its closest competitor. With this concentration of genistein producing better immune responses than the general control or negative control, it can be concluded as an appropriate dosage of genistein to use in this model for further analysis of other individual isoflavones, as well as a disease challenge.

Individual isoflavones did not induce a more prominent innate immune response than the negative control, poly I:C, or the positive control, diclofenac. While this may seem contradictory, poly I:C is, in general, an immunostimulant and has been proven to stimulate chemokines in a manner similar to lipopolysaccharides (LPS) which is commonly used as a negative control. While it is unclear why that scenario occurred in this trial, the number of studies that observed similar outcomes ensure that poly I:C is not exclusively a pro-inflammatory cytokine producer. The isoflavones caused less IL-8 secretion, suggesting a lower stimulation of inflammatory compounds while potentially exerting an anti-inflammatory effect. There were no differences in TEER for treatment or time, while time is typically seen to increase epithelial integrity. This could differ in this trial from the cells having been seeded into the wells heavier and given 5 days instead of 7 to become confluent and start building integrity on the transmembrane cell culture insert. There was no difference caused by treatment either, which is reassuring that the isoflavones may be cultured individually on these cells without degrading them.

CONCLUSION

Genistein in concentrations 150 and 300 μM did not produce a greater innate immune response than LPS; however, 50 μM of genistein was second best and still above the control and negative control responses. There were no overall significant differences in treatment for IL-8 ($P > 0.10$); however, IL-8 secretion was significantly higher in the BLM ($P < 0.001$) and increased over time ($P < 0.001$). Treatment with LPS caused the cells to have greater TEER ($P = 0.0137$) which gradually increased with time ($P = 0.0107$) with the most drastic change between hours 6 and 12. Genistein in a 50 μM concentration is sufficient for further cell culture experimentation while minimizing cost. The remaining isoflavones should be tested at this concentration compared to genistein; however, it is important to note that genistein is the bioactive isoflavone and a mixture of isoflavones should be tested as well to look for interactions.

The individual isoflavones secreted less IL-8 than the controls did, suggesting that a general control should be used during further experimentation to ensure that they still are producing a positive immune response. There was an increase in IL-8 from each treatment over time, which agrees with our previous experiment. While there wasn't a large difference observed in IL-8 concentrations among the controls in this experiment, it gives reason to believe that isoflavones can be cultured on IPEC-J2 cells safely. This gives way to future experiments that can include how cells treated with isoflavones and infected with a pathogen compare to cells treated with isoflavones that have not undergone infection. The TEER results support this statement because there wasn't an increase in intestinal integrity, nor was there a decrease. Another factor of interest in

future studies could be the regulation of MUC-2, the prominent mucin producing gene in cells that could explain how mucosal lining is affected, as well.

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FIGURES

Figure 4.1. IL-8 concentrations in the BLM and AM in Exp. 1 over time expressed as picograms/microliter.

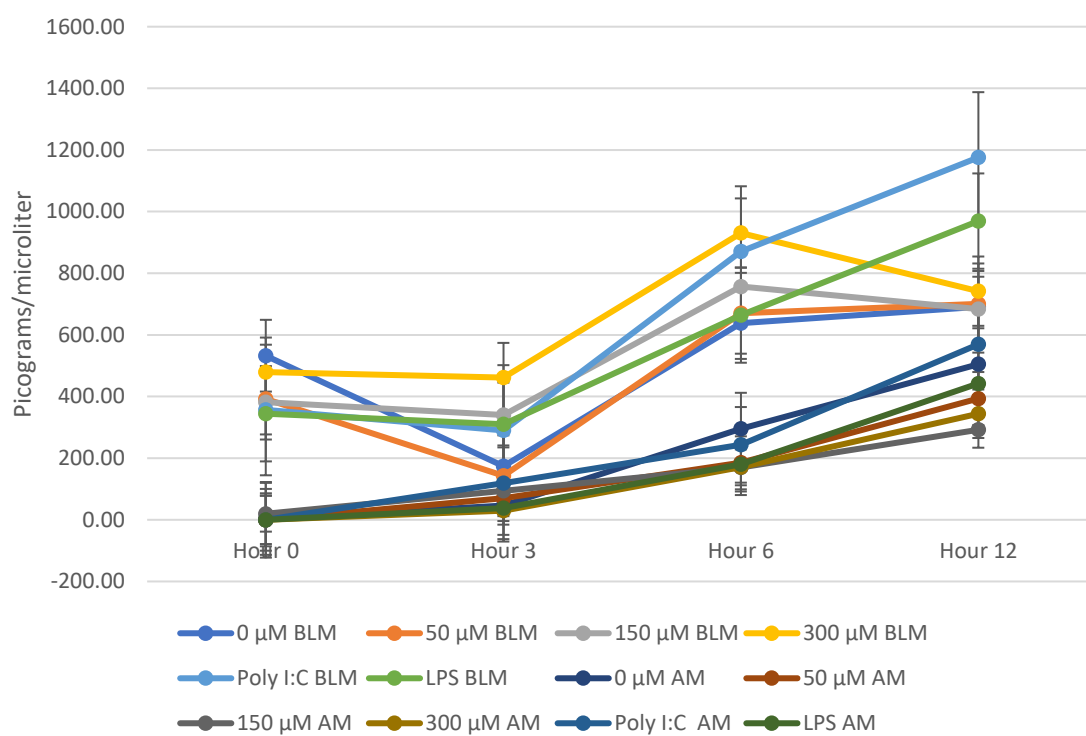


Figure 4.2. IL-8 concentration in the BLM over time in Exp. 1 expressed as picograms/microliter.

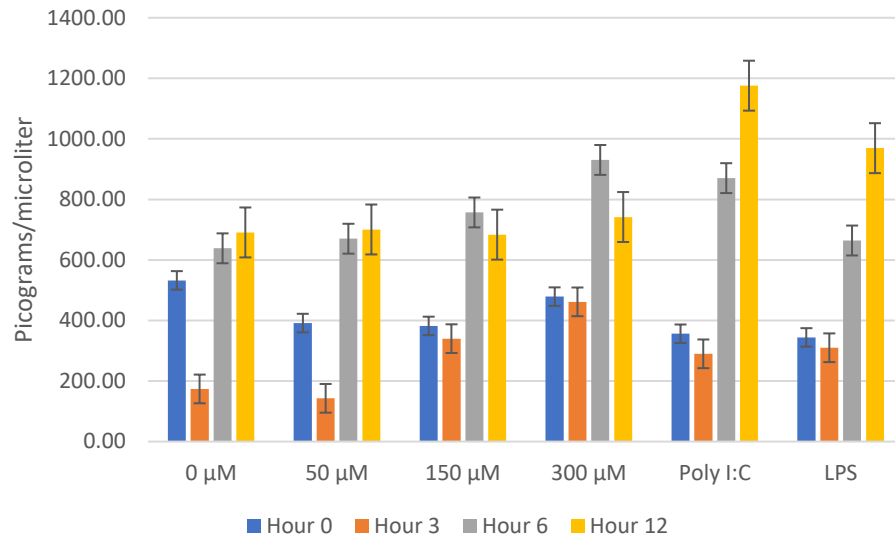


Figure 4.3. IL-8 concentration in the AM over time in Exp. 1 expressed as picograms/microliter.

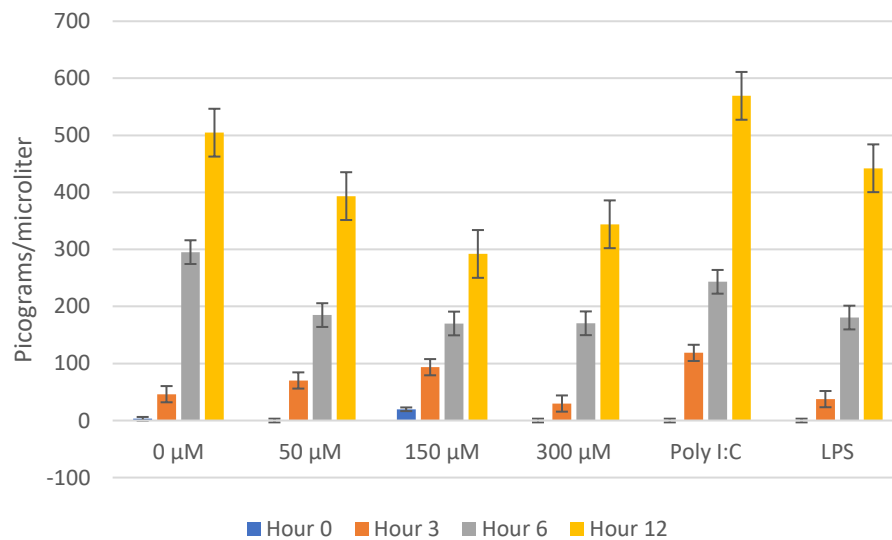


Figure 4.4. Transepithelial electrical resistance of treatments over time in Exp. 1.

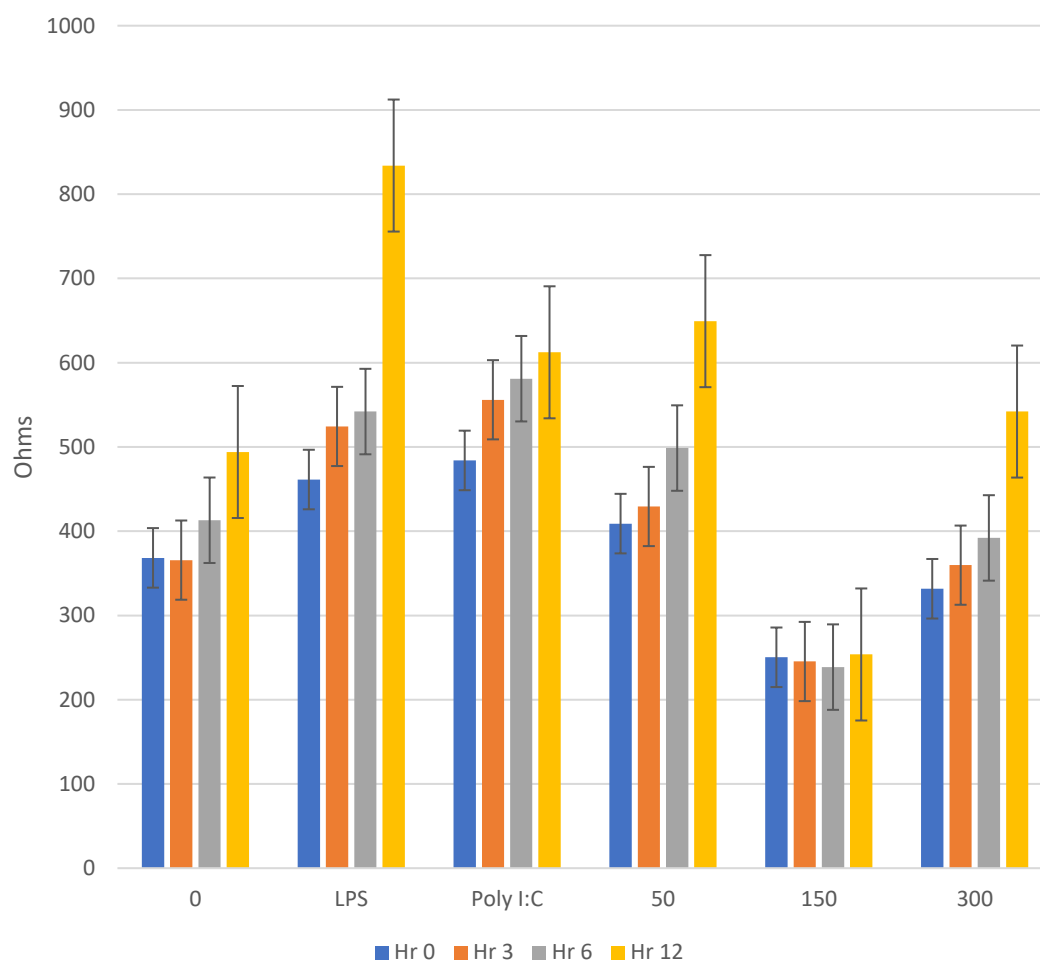


Figure 4.5. IL-8 concentrations in the BLM and AM in Exp. 2 over time expressed as picograms/microliter.

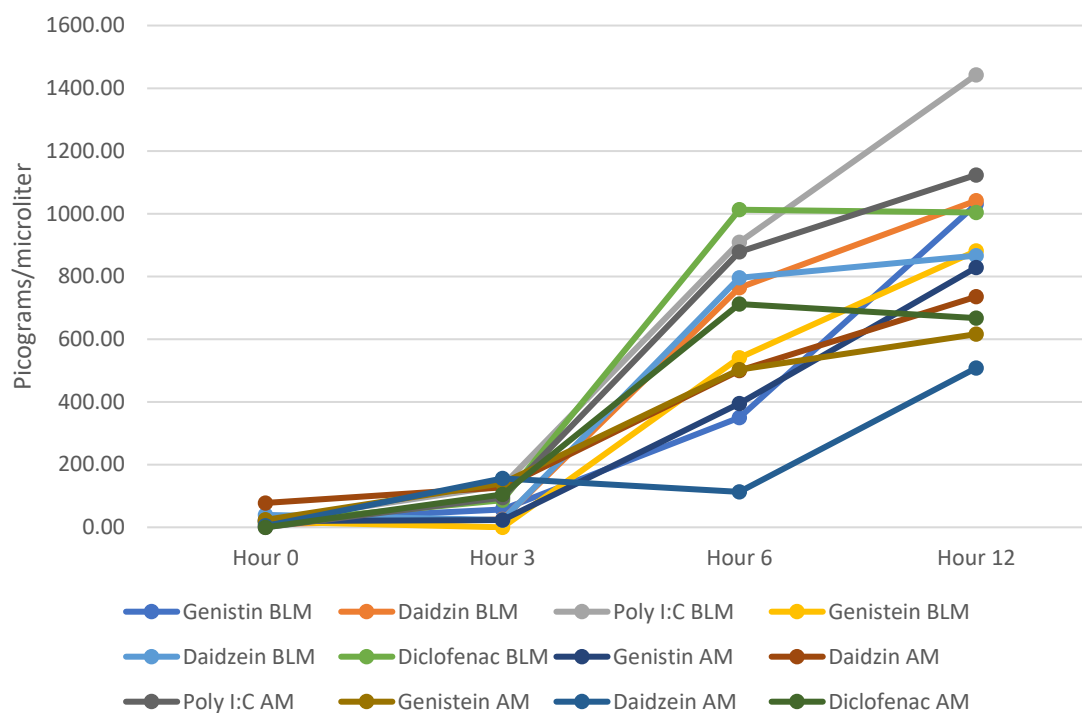


Figure 4.6. IL-8 concentration in the BLM over time in Exp. 2 expressed as picograms/microliter.

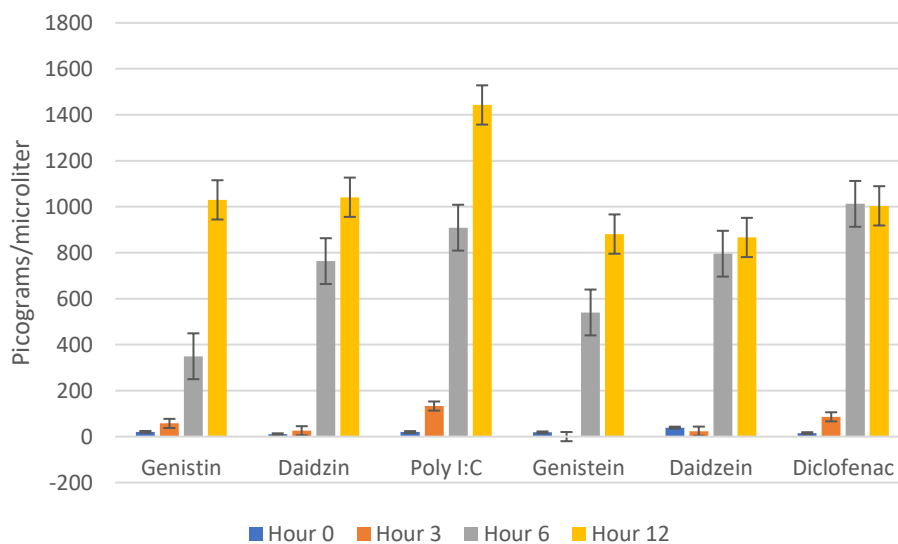


Figure 4.7. IL-8 concentration in the AM over time in Exp. 2 expressed as picograms/microliter.

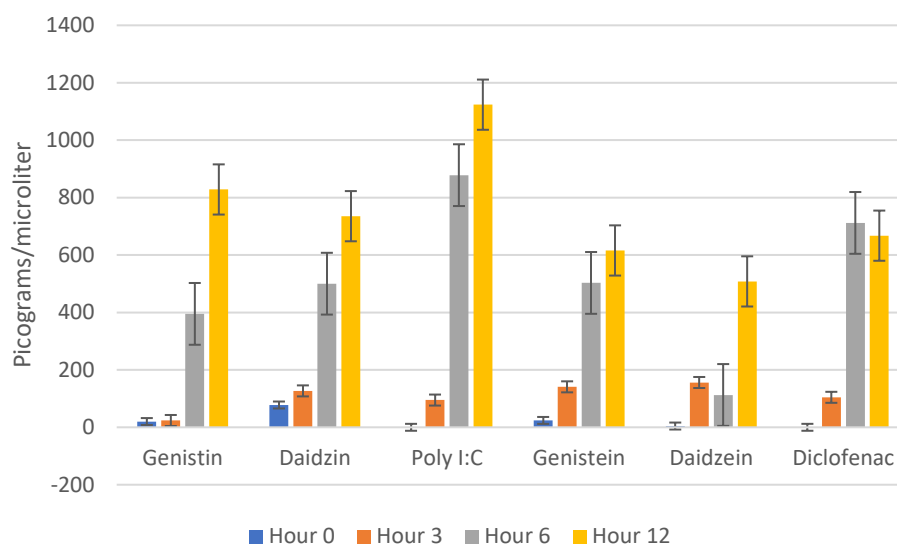
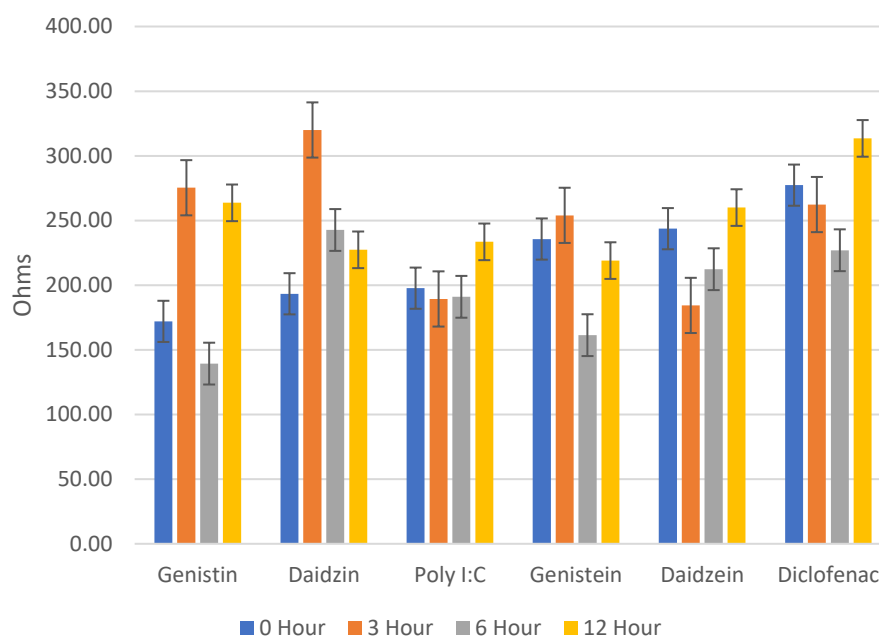


Figure 4.8. Transepithelial electrical resistance of treatments over time in Exp. 2.



ASV Comparison Between Chapter 2 and Chapter 3

When comparing microbiome studies, amplicon sequence variants (ASVs) are commonly used for their reusability, reproducibility, and resolution. In order to establish a baseline microbiome for nursery piglets, metanalysis of studies that sequence GIT samples are possible through the use of ASVs instead of operational taxonomic units because the variation and errors in the DNA sequence are able to be traced to the location of the base pairs. The microbiome data from chapters 2 and 3 were merged and analyzed using *simper*, a function of the *vegan* package in R. This function discriminates species using Bray Curtis dissimilarities and displays the most important species for each group that contribute to at least 70% of the differences between variables.

In chapters 2 and 3, diets were compared and a few bacteria causing differences were *Prevotella*, *Clostridium*, *Lactobacillus*, and *Ruminococcaceae* with most variance occurring as a result of the *Prevotella* abundance. As previously mentioned, *Prevotella* is the most abundant species in the adult GIT and it would be of interest to determine which diets contributed to colonization for this species and why it is the most abundant. *Clostridium* is a prominent butyrate producer, ferments amino acids, and is found in higher abundance in those individuals that are able to produce equol. The comparisons could be done to determine if the diets containing a greater inclusion of isoflavones do have a greater presence of this bacteria. *Lactobacillus* is commonly found in probiotics and assists in carbohydrate fermentation. Chapter 2 explores the effects of varying carbohydrate sources on the microbiome and one or all of these diets could cause greater colonization when compared to chapter 3. *Ruminococcaceae* caused the most variation in microbial composition in chapter 3 and is a common carbohydrate fermenter. There

appears to have been a difference in carbohydrate fermentation among the two studies when analyzing diets. More analysis should be done to determine if the piglets ingesting the varying carbohydrate sources will be more adept to ferment carbohydrates based on the colonization of the species containing the carbohydrate active enzymes.

Sampling location was compared among the studies and it has already been determined that microbial composition varies significantly based on the location the samples were isolated from in both chapters 2 and 3. Again, *Prevotella* was the most prominent species for producing differences, telling that not only does diet affect colonization, but this bacterium is colonizing in one section of the GIT more than the other. *Ruminococcaceae* and the uncultured *Anaerorhabdus* bacterium were at the top of the list for variation. *Ruminococcaceae*, like *Prevotella*, suggests that diet affects the colonization in various parts of the GIT. The presence of *Anaerorhabdus* reminds researchers that there is still much to learn about the structure and function of the gastrointestinal microbiome, but that we are getting closer.

The microbial composition over time was analyzed and *Prevotella* was the bacteria that accounted for the most variation. It is again, the most abundant species in the adult, and the observation that the abundance is changing over time in a nursery piglet is not surprising. *Oscillibacter*, a species known to cause bacterium, fluctuated with time, possibly either by the reduction of the species over time after the piglets recover from the weaning stress, or possibly colonizes more over time as each bacterium establishes its niche in the GIT. The abundance of *Roseburia* also varied with time; however, this species is a commensal bacteria that produces SCFA, especially butyrate, giving it potential to act as a marker of health or serve as a biomarker for symptomatic

pathologies, such as the formation of gallstones. While there are many species that account for differences over time, the diversity of these three account for the theory that the immune system is gradually becoming stronger with the colonization of certain microbes; however, the interaction of the GIT microbiota within itself and with the host could cause functional differences and contributions.

Merging ASVs allows for the reproducibility of studies to be accounted for in research while also contributing to the discovery of microbial composition, the similar species among trials, and which variables (day, diet, sampling location, etc.) cause diversity differences. The contribution of each microbiome trial to a greater study will allow a core microbiome to eventually be established and perhaps even allow individual studies to compare microbial sequences to a large database to determine the differences of their own study to the “norm” and make it easier to locate which bacteria are not typically found in the pig microbiome, but were abundant in their trial. While composition is important, there must also be more research to determine the function of each of the microbial species because just knowing that a species is abundant does not provide researchers with the information of what that actually means for the structure and function of the GIT and what those microbes contribute to the host. Merging these two ASVs is a start for determining microbial similarities in the nursery piglet and is just a fraction of what might soon contribute to a core piglet microbiome.

APPENDIX

DNA Extraction and Sequencing Protocol

DNA Extraction

Kit: Mag Bind Soil DNA kit. 4 x96 preps (Omega)

Set watch bath/incubator to 90°C

Heat elution buffer to 70°C- optional

Prepare ice bucket

Chill centrifuge to 4°C for second centrifugation step

- 1) Place beads in rounded part of a 2 mL safe-lock tube
- 2) Add 0.125-0.25 g of sample to tube. Sample tubes can be frozen until needed to extraction.
- 3) Add 300 uL SLX-Mlus buffer. I use 700 uL for fecal samples to prevent sludge problems.
- 4) Use tissue lyzer to beat bead tubes for 10 min at frequency 20
- 5) Spin down foam by centrifuging at 5000 x g for 2 min at room temp.
Gets the foam off the cap.
- 6) Add 30 uL DS buffer and 2 uL RNase A
 - a) 30 uL x 110 rxns = 3.3 mL
 - b) 2 uL x 110 rxns = 220 uL
 - c) Mix together in a small conical tube
 - d) Dispense 32 uL to each tube
- 7) Vortex to mix thoroughly.
- 8) Place tubes in 90°C water bath for 5-7 min.
- 9) Briefly vortex tubes once during incubation. I do this after 3.5 min.
- 10) Centrifuge 5000 x g for 10 at room temp.
- 11) Transfer 1000 uL supernatant into a new set of 1.5mL tubes.
 - a) You likely won't have this much. Don't be afraid to transfer cloudily liquid.
- 12) Add 67 uL of SP2 buffer and 67 uL cHTR reagent
 - a) Warm up cHTR to 37°C to make sure there are no solids in the bottle. Put back in fridge after use.
 - b) Solution will be very thick to add. Sanitize a razor blade and cut the tip off a pipette tip for use.
- 13) Vortex to mix.
- 14) Let sit on ice for 5 min.
- 15) Centrifuge at 5000 x g for 10 min at 4°C.
- 16) Transfer the supernatant into a new tube. You may have to take some sludgy stuff.
- 17) Can leave at this step for overnight.

Preparation of nucleic acids – do prior to extraction

- 18) Make 100 mL of 70% EtOH (make fresh everytime)

- 19) Make 10 mM sodium acetate
 - a) $\text{g needed} = \text{MW (g/mol)} \times \text{volume needed (L)} \times \text{final conc (M)}$
 - b) $82.03 \text{ g/mole} \times 0.05 \text{ L} \times 0.01 \text{ M} = 0.041 \text{ g}$ measured into 50 mL ddiH₂O
- 20) Get ice bucket
- 21) Put EtOH and isopropanol into ice bucket
- 22) Make 10 mM Tris at pH 8
 - a) Tris base = 121 g/mol
 - b) To make 1 L
 - i. $10 \text{ mM} = 0.01 \text{ M}$
 - ii. $(0.01 \text{ mol/L}) \times (121 \text{ g/mol}) = 1.21 \text{ g/L}$
 - iii. Sterilize by autoclave
 - iv. Let cool to room temp
 - v. pH to 8 (for 250 mL I added approximately 30 uL, but adjust as needed)

Note: pH solution prior to autoclaving if you are worried about sterility.
pH shouldn't change much.
- 23) Add 10 mM of sodium acetate to each lysate tube, vortex gently, and incubate on ice for 5 min (solution is to 0.2X of beginning volume)
 - a. $350 \text{ uL} \times 0.2 = 70 \text{ uL}$ added, I would just add 70 uL regardless of volume
- 24) Centrifuge tubes at 16,000 x g for 15 min at 4°C.
- 25) Transfer supernatant into new 1.5 mL tube
 - a. If less than 650 uL transfer all to one tube. There likely won't be more than 600 uL
- 26) Add one volume (same amount as supernatant from previous step) of isopropanol to supernatant tube and vortex
 - a. I added 300-400 uL
- 27) Incubate tubes on ice for 30 min
- 28) Centrifuge at 4°C for 15 min at 16,000 x g
- 29) Pipette out supernatant and discard (you can just decant)
- 30) Wash nucleic acid pellet with 500 uL ice cold 70% EtOH and gently vortex
 - a. Removes residual salt
- 31) Centrifuge at room temp for 2 min at 13,000 x g
- 32) Decant wash and dry pellet for 3 min
- 33) Dissolve the nucleic acid pellet in 450 uL of Tris (10 mM, pH 8) and transfer to a deep well plate (DWP).
 - a. Its best to let samples set at 4°C for a few hours or overnight. (I do this in the tubes)
 - b. There may be a slight pellet so just mix gently and transfer to DWP. Mix with pipette.
 - c. Verify samples match paper chart
 - d. Samples or DWP can be frozen until King Fisher step is done.

King Fisher Isolation

- 34) Add 0.5 volumes of XP2 buffer, 10 uL Mag-Bind Particles CND, and 5 uL Binding Enhancer to DWP containing DWP. (I do this prior to putting samples in DWP.)

- a. The XP2 buffer, Mag-Bind Particles, and Binding Solution can be prepared in a Master Mix.
- 225 uL x 110 = 24,750 uL = 24.75 mL XP2 buffer
 - 10 uL x 110 = 1100 uL = 1.1 mL Mag-Bind CND
 - 5 uL x 110 = 550 uL Binding Enhancer
 - Add 240 uL of this solution to each well of lysate plate
- 35) Add 300 uL XP2 buffer to a new DWP, 33 mL
- 36) Add 300 uL VHB buffer to a new DWP, 33 mL
- 37) Add 400 uL SPM Wash buffer to a new DWP, 44 mL
- 38) Add 400 uL SPM Wash buffer to a new DWP, 44 mL
- 39) Add 130 uL Elution buffer to a new DWP, 14.3 mL
- 40) Place plates on Kingfisher in correct areas when prompted by machine using protocol Omega_noH2O
- Takes 30 min
 - Make sure to have an additional sterile DWP and tip comb every time.
 - DWP for tip comb can be reused, just place silver tape on it
- 41) Save samples plate and elution plate and cover with silver tape and freeze.
- 42) Transfer 50 uL of elution into a 96 well plate for downstream analysis

PCR Primer Step

- 43) Thaw elution plate and bacterial primer plate on ice or in fridge. Make sure primer plate number matches your plate number (1 for 1, 2 for 2, etc.)
- 44) Remove Terra Box from freezer to thaw.

PCR recipe

Recipe	1 rxn	100 rxn
Terra Buffer	12.5 uL	1250 uL = 1.25 mL
Terra Polymerase	0.5 uL	50 uL
Primer	1.0 uL	These are individual per well
H2O	9.0 uL	900 uL = 0.9 mL
DNA	2.0 uL	200 uL
Total	25.0 uL	2500 uL = 2.5 mL

- 45) Spin elution and primer plates in centrifuge at 3000 rpm for 1 min
- 46) Add reagents to PCR plate
- Master mix = Buffer + polymerase + H2O = 22 uL, can place in
 - Primer = 1 uL
 - DNA = 2 uL
 - Seal with a plate cover, make sure its well sealed
 - Spin PCR plate down.

- 47) Turn on PCR machine and place plate in holder

- 48) Use the "Illumina PCR 25c" protocol, 25 uL of total reaction

Stage	1	2	2	2	3	3
Step	1	1	2	3	1	2
Temp, °C	98	98	55	68	68	4

Time, m:ss	3:00	0:30	0:30	0:45	4:00	∞
Repetition	x1	x25			x1	

49) When PCR is finished, spin down plate. Plate can be covered with silver tape and frozen or proceed to agarose gel.

DNA Agarose Gel Electrophoresis

- 50) Make an 1.5% agarose gel with 2 50-well combs
 - a. 250 mL TBE buffer + 3.75 g agarose
 - b. Microwave carefully until agarose has dissolved, make sure to avoid boil over
 - c. Temper media, add 12.5 uL of ethidium bromide and mix
 - d. Prior to pouring into gel casting frame. Add combs.
 - e. Let gel polymerize for an hour
- 51) Spin down PCR plate at 3000 rpm for 1 min.
- 52) Add 5 uL of PCR product to PCR plate and 3 uL of Xylene loading dye to each well
- 53) Seal primer plate with silver tape and freeze for later use.
- 54) Spin down loading dye plate.
- 55) Remove 50 kb ladder from freezer
- 56) Remove gel from casting frame and place in running box with buffer, remove combs and make sure buffer floods all wells.
- 57) Add ladder to the first well on each well set.
- 58) Use multichannel to load samples into wells.
- 59) Plug in apparatus to power source and run at 120 V, 75 ?amps, and 60-75 mins.
- 60) Remove gel from buffer and place in gel doc to image gel.
 - a. Take a picture and double check bands. Clear bands are good. Smearing is boo bad.

Normalization Step

- 61) Make sure PCR Amplified plate is thawed and spun down.
- 62) Add 20 uL of sample to a 96 well Binding plate (brand name), do not put tips in bottom of wells.
- 63) Cover and spin down.
- 64) Turn on epMotion robot.
 - a. 96 well plate and solutions come from Charm Biotech 96 PCR Purification and Normalization kit
 - b. Place 96 well plate on area B1. (The spot that holds a plate.)
 - c. Place new tip boxes on A2 and B2.
 - d. Place cartridge containing buffers on C2.
 - i. Solutions will be added to epMotion 30 mL Reservoirs.
 - ii. Binding solution, leftmost spot, and minimum of 2.125 mL needed
 - iii. Washing buffer, next spot, minimum of 9.805 mL needed.
 - iv. Elution buffer, next spot, minimum of 2.125 mL needed.
 - v. Reservoir covers should be taken off and placed under the machine for coverage.

- e. Empty used tip bin if not done already. Keep an eye on this to avoid having tips get stuck on the robot pipette and causing problems.
 - f. Load “Normalizationnewkit50” protocol on computer.
 - i. Add 20 uL of binding solution to all wells.
 - ii. Incubate for 60 min.
 - iii. Dump out liquid and spin plate upside down on a paper towel for 1 min at 3000 rpm. Place plate back in the robot.
 - iv. Add 50 uL of washing buffer, incubate 30 sec, repeat step iii. Put a new rack of tips in A2.
 - v. Repeat step iv.
 - vi. Wait 2 min, add 20 uL of Elution buffer.
 - g. Click “Run” tab, check “HEPA” box, well volume should be 20 uL, make sure buffers meet minimum quantity. Add more volume for column next to minimum.
 - h. Robot might not recognize tip boxes, make sure tips are there and ignore warning. Robot will also complain solution cartridge, click “Ignore”
 - i. Door can lifted to halt robot at anytime, close to resume.
- 65) Cover plate, vortex for 30 sec, spin down.

Pooling Sample libraries

- 66) Use epMotion robot again. Select “pooling_10uL_VWRtips” protocol.
- a. Protocol will take 10 uL from each well and pool it in the 1.5 mL tube.
- 67) Place a new box of tips in A2. Dump used tip bin.
- 68) Place Blunting cartridge in C2, add clean 1.5 mL tube to the A1 position (top left corner)
- 69) Click “Run” tab. Check HEPA box, click through “Next” steps until start.
- 70) When finished, cover plate with silver tape and freeze. Label tube and freeze or continue to purification step.

Purification of DNA library

- 71) Follow protocol for “PCR clean-up “ of NucleoSpin Gel and PCR Clean-up kit. Use 1:2 dilution.
- a. Adjust DNA binding condition
 - For very small sample volumes < 30 uL adjust the volume of the reaction mixture to 50-100 uL with water.
 - Mix 1 volume of sample with 2 volumes of Buffer NTI
 - 960 uL of DNA + 1920 uL of Mix, Mix is 1:2, 640 NTI buffer + 1280 PCR H2O
 - b. Bind DNA
 - Place a Nucleospin Gel and PCR Clean-up Column into a Collection Tube (2 mL) and load up to 700 uL sample.
 - Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the collection tube.
 - Load remaining sample if necessary and repeat the centrifugation step.

c. Add 700 uL Buffer NT3 to the NucleoSpin Gel and PCR Clean-up Column. Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the collection tube.

Repeat previous washing step to minimize chaotropic salt carry-over and improve A_{260}/A_{230} values.

d. Dry silica membrane

Centrifuge for 1 min at 11,000 x g to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube.

e. Elute DNA

Place the Nucleospin Gel nad PCR Clean-up Column into a new 1.5 mL microcentrifuge tube. Add 15-30 uL Buffer NE and incubate at room temperature (18-25°C) for 1 min. Centrifuge for 1 min at 11,000 x g.

72) Label tube with plate number and freeze.

Quantification of DNA library

73) Use DeNovix kit and reader. You must use DeNovix tubes for procedure.

74) Making working solution (WS)

a. Make sure buffer and dye are at room temp when used. You can take an aliquot of the buffer so you don't have to get the whole bottle to temp.

b. 200 uL buffer + 2.0 uL dye for each library and standard. Add an extra 0.5 to provide room for pipetting error. (2 standards + 4 library + 0.5 extra = 6.5 libraries)

c. 200 uL buffer x 6.5 = 1300 uL buffer, 2.0 uL dye x 6.5 = 13.0 uL dye

75) Add working solution to tubes labeled for standards and samples.

a. Standards need to be made each day for quantification.

b. Don't add standard or sample library until ready to proceed.

c. 190 uL WS + 10 uL of 0 standard

d. 190 uL WS + 10 uL of 25 standard

e. 199 uL WS + 1 uL sample library (Make one for each sample library)

76) Follow procedure for standards and samples, one at a time:

a. Launch Fluoro dsDNA app using DeNovix fluorometer.

b. Use drop down menu to select "DeNovix dsDNA Ultra High Sensitivity Assay"

c. Select "Preconfigured 2 standards" and then choose "Generate New Standard Curve"

d. Add 0 standard to tube.

e. Spin briefly on mini centrifuge to ensure samples are mixed together.

f. Time for 5 min

g. Place tube in Fluorometer slot and close lid, tap "Measure" to measure.

h. Add 25 standard to tube and repeat steps e, f, and g.

i. After both standards have been run, tap the "Samples" button.

- j. Add sample library DNA to respective tube and repeat steps e, f, and g. Write down concentration.
 - k. Repeat until all libraries have been run.
- 77) Enter concentrations into Excel file “Library Dillution Calculator Illumina”. This will convert pg/mL to nM.
- 78) Determine dilutions needed to created 4 nM libraries for pooling. (Need to add to this once I have hit this step.
- a. $C1V1=C2V2$

Aligent High Sensitivity DNA Kit Quick Start Guide For Bioanalyzer Chip

Preparing the Gel-Dye Mix

1. Allow high sensitivity DNA dye concentrate (blue) and high sensitivity DNA gel matrix (red) to equilibrate to room temperature for 30 min.
2. Add 15 uL of high sensitivity DNA dye concentrate (blue) to a high sensitivity DNA gel matrix vial (red).
3. Vortex solution well and spin down. Transfer to spin filter.
4. Centrifuge at 2240 g +/- 20% for 10 min. Protect solution from light. Store at 4°C. Use prepared gel-dye mix within 6 weeks of preparation

Loading the Gel-Dye Mix

1. Allow the gel-dye mix to equilibrate to room temperature for 30 min before use.
2. Put a new high sensitivity DNA chip on the chip priming station.
3. Pipette 9 uL of gel-dye mix in the well marked G with white lettering and a black circle around it.
4. Make sure that the plunger is positioned at 1 mL and then close the chip priming station.
5. Press plunger until it is held by the clip.
6. Wait for exactly 60 s then release clip.
7. Wait for 5 s, then slowly pull back the plunger to the 1 mL position.
8. Open the chip priming station and pipette 9 uL of gel-dye mix in the wells marked G with black lettering, white surrounding.

Loading the Marker

1. Pipette 5 uL of marker (green) in all sample and ladder wells. Do not leave any wells empty.

Loading the Ladder and Samples

1. Pipette 1 uL of high sensitivity DNA ladder (yellow) in the well marked with a ladder.
2. In each of the 11 sample wells pipette 1 uL of sample (used wells) or 1 uL of marker (unused wells).
3. Put the chip horizontally in the adapter and vortex for 1 min at the indicated setting (2400 rpm).
4. Run the chip in the Agilent 2100 Bioanalyzer instrument within 5 min.

Sodium Acetate and Ethanol DNA Library Precipitation

1. Add 1/10 volume sodium acetate (3M, pH=5.2).

2. Add 2.5X volume (calculated after addition of sodium acetate) of at least 100% isopropanol.
3. Add 1.0 uL Carrier RNA from Qiagen kit to tube.
4. Incubate at -80°C overnight.
5. Centrifuge at >14,000 x g for 30 min at room temperature to 4°C.
6. Discard supernatant carefully, making sure the DNA pellet (may not be visible) is not discarded.
7. Rinse with cold 50 uL 70% EtOH.
8. Centrifuge for 15 min at 4°C.
9. Discard supernatant and dissolve pellet in TE or EB. Make sure buffer comes in contact with the whole surface of the tube since a significant portion of DNA may be deposited in the walls instead of in the pellet.
*** Don't resuspend pellet until sequencing, trying to minimize library concentrate degradation.

BluePippin Purification Protocol – 1.5% Agarose Gel Cassette

1. Prepare DNA samples
 - a. Bring DNA sample up to 30 uL with TE.
 - b. Bring loading solution to room temperature.
 - c. For each sample, combine 30 uL of DNA sample with 10 uL of leading solution/marker mix (labeled K).
 - d. Mix samples thoroughly (vortex mixer). Briefly centrifuge to collect.
2. Program a Protocol
 - a. In the Pippin Prep Software, go to the Protocol Editor Tab.
 - b. Click “Cassette” folder, and select “**1.5% DF Marker K**”.
 - c. Select the collection mode for each lane (usually “Tight” or “Range”, and enter the size selection parameters.
 - d. Click the “Use Internal Standards” button.
 - e. Make sure the “Ref Lane” values match the lane numbers.
 - f. Press “Save As” and name and save the protocol.
3. Calibrate the Optics with the Calibration Fixture
 - a. Place the calibration fixture onto the optical nest. Close the lid.
 - b. Press “CALIBRATE” to launch the calibration window.
 - c. Enter 0.80 in the “Target I ph, mA” field. Press “CALIBRATE” button in the window, and when complete press “EXIT”.
4. Inspect the Gel Cassette
 - a. Remove the cassette from the foil packaging.
 - b. Inspect the levels of buffer in all buffer reservoirs. Reservoirs should be nearly full. If the buffer level in any reservoir appears less than 50% full, fill with spare buffer.
 - c. Inspect the gel columns. Look for obvious breakage of the agarose column in each channel. If there is obvious breakage, do not use the lane. Remaining lanes can be used.
 - d. Inspect for bubbles due to separation of agarose from bottom of cassette in the region used for optical detection of DNA. If a bubble is observed, do

not use the lane. A bubble on the top of the gel column will not affect detection.

5. Prepare the Cassette for Loading

- a. Dislodge bubbles from behind the elution wells. Tilt the cassette sample well side down, to release the any trapped bubbles behind the elution modules.
- b. Place Cassette into the optical nest. Keep the cassette slightly tilted down so that the bubbles in the elution reservoirs don't return to the area behind the elution modules. Be sure the cassette is fully seated into the bottom of the nest to ensure proper optical alignment.
- c. Remove the white tabbed adhesive strips from the cassette. Place one hand on the cassette, and hold it firmly in the nest. Grab the white tabs of the tape and pull the strips firmly and slowly toward the front of the BluePippin until they are removed.
- d. Remove buffer from all elution modules and replace with 40 uL of fresh electrophoresis buffer. Make sure that the pipette tips extend all the way to the bottom of the elution modules without sealing the elution port opening. Test tip fit using the empty rinse cassette supplied with the instrument.
- e. Seal the elution wells with the adhesive tape strips. Tape for sealing the elution wells are supplied with cassette packaging. Place tape over the elution wells and rub firmly to fix the tape in position.
- f. Check the buffer level in the sample wells. Immediately prior to loading, sample wells should be completely filled to the top with buffer. If any wells are underfilled, top them with additional buffer.
- g. Perform the continuity test. Close the lid and press the "Test" button located in the lower right area of the Main screen. The test routine runs automatically and measures the current in each separation and elution channel. And should return a "PASS" for each separation and elution channel. The cassette temperature must be above 17°C(62°F).

6. Load Samples

- a. Re-check the buffer level in the sample wells. Make sure that sample wells are completely full to the top with electrophoresis buffer. Fill with additional buffer, if necessary.
- b. Remove 40 uL of buffer from the first sample well, and load 40 uL of sample into that well. Take care to not pierce the agarose with the pipette tip. There is gel on all sides and bottom of the sample well. In addition, there is an agarose "chimney" surrounding the top of the sample well that protrudes up through the cassette cover. When removing buffer, some users find it useful to immerse the pipette tip just below the surface of the buffer and follow the liquid level down with the tip as the buffer is removed. When buffer removal is completed, there will be ~30 uL of buffer left in the well. When adding sample, place tip of pipette just below the surface of the buffer, and follow the liquid level up with the tip as the well fills. Don't be concerned if the sample well slightly overfills. The

density of the sample will allow it to sink before it can flow out of the well.

- c. Repeat step b for the remaining four wells.
7. Run
 - a. Close the lid, go to the Main Tab, and make sure the proper protocol is loaded in the “Protocol Name” field.
 - b. Press “START”. The run will automatically stop when every collection is complete.
8. Collect Fractions
 - a. Remove samples using a standard 100-200 uL pipette. Samples will be in a Tris-TAPS buffer at a volume of 40 uL. Samples should be suitable for amplification. Do not let samples remain in cassette overnight.
 - b. Remove cassette and dispose of properly. Do not keep used cassettes in the Pippin with the cover closed. Humidity from the cassette may cause damage to electrodes.

NextSeq System Denature and Dilute Libraries Guide

1. Denature Libraries
 - a. Combine 5 uL of DNA library and 5 uL 0.2 N NaOH in a microcentrifuge tube.
 - b. Vortex briefly and then centrifuge at 280 x g for 1 minute.
 - c. Incubate at room temperature for 5 minutes.
 - d. Add the following volume of 200 mM Tris-HCl, pH 7.
 - e. Vortex briefly and then centrifuge at 280 x g for 1 minute.
2. Dilute Denatured Libraries to 20 pM.
 - a. Add the following volume of prechilled HT1 to the tube of denatured libraries.
 - i. 985 uL for 4 nM
 - ii. The result is a 20 pM denatured library.
 - b. Vortex briefly and then centrifuge at 280 x g for 1 minute.
 - c. Place the 20 pM libraries on ice until you are ready to proceed to final dilution.

Speed-Vacuum DNA Concentration

1. Using Denovix kit, determine concentration of DNA present in elution buffer and how much elution buffer needed to achieve optimal concentration.
2. Place microcentrifuge tube containing library in Speed-Vacuum with tube cap open and a balance and close the lid.
3. Turn on centrifuge portion of vacuum followed by the vacuum pump.
4. Monitor amount of fluid present in library until desired concentration is achieved.
5. Shut off vacuum and centrifuge.
6. Check concentration with Denovix again.

MiSeq Library Preparation and Sequencing

1. Remove a 500 cycle reagent cartridge from the -20 °C freezer. Place in room temperature water bath for one hour. Place HT1 buffer tube in 4 °C fridge.
2. Copy sample sheet to sample sheet folder on MiSeq. Rename sample sheet to match barcode of reagent cartridge.
3. When the reagent cartridge has thawed, dry bottom with paper towel. Invert the cartridge repeatedly to check each well is thawed. This also served to mix the reagents. Place in hood.
4. Thaw library, PhiX, and sequencing primers. Check to make sure HT1 is thawed.
5. Place 3 uL of the Read 1 Sequencing Primer(s) into a clean PCR tube. Repeat in separate tubes for the Index Primer(s) and Read 2 Sequencing Primer(s).
6. Using a 1000 uL pipette tip, break the foil over wells 12, 13, 14, and 17.
7. Use an extra long 100 uL tip with the pipettor set on 75 uL to transfer the 3 uL of Read 1 Sequencing Primer to the bottom of well 12 and pipette 10X to mix. Repeat this process spiling the Index primer into well 13 and the Read 2 Sequencing Primer into well 14.
8. Prepare a fresh dilution of 0.2N NaOH.
9. To a 1.5 mL tube add 10 uL of library, and 10 uL of 0.2N NaOH. To a separate tube add 2 uL PhiX, 3 uL PCR grade water, and 5 uL of 0.2N NaOH. Vortex both tubes to mix and spin for 1 minute at 400rcf.
10. Allow the tubes to incubate at room temperature for 5 minutes. Immediately add 980 uL of HT1 to library tube and 990 uL HT1 to the PhiX tube.
11. Use HT1 to dilute both the library and PhiX to 10pM. For a 5% PhiX run, combine 950 uL of 3.5pM Library and 50 uL PhiX in a final tube. Vortex. Load 600 uL of this solution into well 18 on the reagent cartridge. See example below:
 - a. $(1.45 \text{ nM library} \times 10 \text{ uL}) + (0.2\text{N NaOH} \times 10 \text{ uL}) + 980 \text{ uL HT1} = 14.5 \text{ pM Lib, } 0.002\text{N NaOH}$
 - b. $(14.5\text{pM lib} \times 241.38 \text{ uL} + 758.62 \text{ uL HT1} = 3.5\text{pM lib, } 0.00048\text{N NaOH}$
 - c. $[(10\text{nM PhiX} \times 2 \text{ uL}) + 3 \text{ uL H}_2\text{O}] + (0.2\text{N NaOH} \times 5 \text{ uL}) + 990 \text{ uL HT2} = 20\text{pM PhiX, } 0.001 \text{ NaOH}$
 - d. $(20\text{pM PhiX} \times 175 \text{ uL}) + 825 \text{ uL HT1} = 3.5 \text{ PhiX, } 0.0000175\text{N NaOH}$
 - e. $(3.5\text{pM Lib} \times 950 \text{ uL}) + (3.5\text{M PhiX} \times 50 \text{ uL}) = \text{solution loaded}$
 - f. Solution loaded is 3.5pM overall with a 3.325 pM library concentration, 0.175 PhiX concentration, and 0.0000457N NaOH
12. Set reagent cartridge aside. Unbox flow cell and PR2 bottle.
13. Thoroughly rinse the flow cell with Milli-Q water. Carefully dry by blotting with lint free wipes. Give special attention to the edges and points of intersection between the glass and plastic.
14. We a new wipe with 100% alcohol and wipe the glass on both sides avoiding the rubber intake ports.
15. Visually inspect the flow cell to ensure there are no blemishes, particles, or fibers on the glass.

16. Follow the screen instructions and load the flow cell, reagent cartridge, and PR2 bottle. Empty and replace the waste bottle.
17. Ensure the machine recognizes the correct sample sheet and the run parameters are correct.
18. Wait for the MiSeq to perform its pre-run check, and press start.